2018

Evaluating The Resistome And Microbial Composition During Food Waste Feeding And Composting On A Vermont Poultry Farm

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EVALUATING THE RESISTOME AND MICROBIAL COMPOSITION DURING FOOD WASTE FEEDING AND COMPOSTING ON A VERMONT POULTRY FARM

A Thesis Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Animal Science

May, 2018

Defense Date: March 23, 2018
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ABSTRACT

While commonly thought of as a waste product, food scraps and residuals represent an important opportunity for energy and nutrient recapture within the food system. As demands on production continue to increase, conservation of these valuable resources has become a priority area. In the wake of new legislation in Vermont, Act 148, the Universal Recycling Law, the fate of microbial species in food waste, scraps and residuals is increasingly important. The presence of antimicrobial resistance genes in all types of foods calls for an increased need to estimate risk of antibiotic resistance transfer and maintenance across all segments of food production and distribution systems, from farm to fork. Specifically, the fate of antibiotic resistance genes (ARGs) in these co-mingled food wastes has not been sufficiently characterized; as legislative programs increase in popularity, surveillance of these materials is pressing and should be documented to assess the risk and potential measures for mitigation and management as we approach commercial scales of implementation

Previous studies have relied on a combination of targeted techniques, such as 16S rRNA sequencing and qPCR on a specific subset of ARGs; however, these may not cover the full extent of resistance or microorganisms of concern in any given sample. As sequencing technologies improve and costs continue to drop, more comprehensive tools, such as shotgun metagenomic sequencing, can be applied to these problems for both surveillance and novel gene discovery. In this study, we leveraged the increased screening power of the Illumina HiSeq and shotgun metagenomic sequencing to identify and characterize ARGs, microbial communities, and associated virulence factors of food scraps, on-farm composts, and several consumer products. Isolates were also screened for antibiotic resistance to demonstrate the functionality of ARGs identified.

The resistome, microbiome, and virulence genes were characterized in all samples. Fifty unique ARGs were identified that spanned 8 major drug classes. Most frequently found were genes related to aminoglycoside, macrolide, and tetracycline resistance. Additionally, 54 distinct virulence factors and 495 bacterial species were identified. Virulence factors were present across the farm setting and mainly included gene transfer mechanisms, while bacteria clustered distinctly into site and farm, as well as separate on farm niches. The relationship between these categories was also assessed by both Pearson correlation and co-inertia analysis, with the most significant relationship being between ARGs and virulence factors ($P = 0.05$, RV = 0.67). While limited in this study, these patterns reinforce the finding that spread of antibiotic resistance genes may be dependent on the virulence factors present enabling transfer, rather than total microbial community composition.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. John Barlow for his willingness to entertain new ideas and constant brainstorming sessions that resulted in this project coming to fruition. It's truly been a pleasure and invaluable learning experience.

I would also like to thank my committee members, Dr. Melissa Pespeni and Dr. Jana Kraft for their support and mentorship. Additional thanks and gratitude must go to Tom Gilbert and all the members of Black Dirt Farm for their participation. Finally, to the members of the Barlow lab past and present and other graduate students, especially Mital Pandya, Tatjana Sitt, and Robert Mugabi for convincing me to stay in the lab and continue this process.

A special thanks goes to my family and friends especially my parents, Annie, and Birgit, for indulging my one-sided rants, supporting me throughout this process, and taking the ever-changing trajectory of my work in stride.

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1.1. Food Wastes from Table to Farm

As two of the greatest public health challenges faced today, food waste and antimicrobial resistance are economically and environmentally costly. The pressure to feed the world's ever-growing population while keeping costs low manifested in historical use of antimicrobials as growth promoters in animal agriculture, often described as primary contributor to the spread of antibiotic resistant bacteria (Chang et al. 2015). The spread of resistant pathogens and antibiotic resistance genes (ARGs) has been inexorably linked to the contamination and movement of agricultural products. Antimicrobial resistant bacteria (ARB) have been isolated from a variety of commercial products, such as meats (Doyle 2015), dairy products (Silveira-Filho et al. 2014; Kevenk and Gulel 2016), and even raw produce (Bezanson et al. 2008). Reports detailing the impacts of commercial agriculture and food safety have given rise to a social movement that goes by many names. Farm-to-table, farm-to-fork, or locavore; all have become go-to terms for consumers, producers, legislators, and researchers. Even the U.S. Center for Disease Control (CDC) has co-opted this language in materials detailing the impacts of antimicrobial resistance on food safety (CDC 2018).

As the estimated global costs of antimicrobial resistance are predicted to hit $100 trillion annually by 2050 (Adeyi 2017), efforts to produce food without extensive use of antibiotics as growth promoters, limit transportation and preservation of foods, and reduce wastes and residuals all fall within the scope of "farm-to-table" style eating. Eating local, improving consumer access, and building communities around food

production are all components of the farm-to-table movement (Massey 2015), which may strengthen the awareness and relationships necessary for the increased price associated with improved antimicrobial stewardship.

In addition to concerns over the spread of antimicrobial resistance, rising global food waste is a major contributor to global public health concerns. Global food waste has risen to 1.3 billion metric tonnes a year (FAO 2018), with approximately 133 billion pounds attributed to the U.S. alone (USDA 2018). Efforts to conserve these resources will bring human-derived food wastes back into the agricultural sector at unprecedented rates. Several state and national governing bodies have launched efforts to improve conservation, such as Act 148 in the state of Vermont or the Food Recovery Challenge issued by the Environmental Protection Agency (EPA). In both instances, diversion of food scraps and residuals to agricultural production and composting is a major component food recovery.

The return of these co-mingled wastes, the table-to-farm portion of the food system, has garnered less attention however; especially in the assessment of food safety and potential further spread of antimicrobial resistance. In recent years, diversion of food wastes to agriculture as animal feed or substrate for composting operations has presented an invaluable opportunity to recapture energy and nutrients that may otherwise end up in landfills. It also gives farmers a chance to save on feed costs as well as capture additional income from selling these value-added products and hauling fees. Finally, it reduces the critically high methane emissions from landfills, contributing to approximately 18% of total U.S. emissions (EPA 2014).

The aim of this work is to examine the impacts of food wastes on the potential spread of antimicrobial resistance when used as poultry feed and substrate for compost both on the farm and upon its return to consumers. Legislative mandates and producer incentives have increased the popularity of this management strategy, but little information about the potential risks of antimicrobial resistance transfer are known at this time. Previous work has identified ARB and ARGs in many of the materials that will make-up diverted food wastes, yet specific knowledge of the abundance and identity of these genes throughout the food waste composting cycle is lacking. Additionally, new technologies, such as shotgun metagenomic sequencing, have made surveillance of these materials more accessible and may help shed light on the fate of ARB and ARGs throughout the food scrap composting process.

1.2. Food Waste

Global estimates of food waste have reached staggering proportions; current FAO estimates state that at least one third of the food produced globally is not consumed (FAO 2011). In the U.S. alone it's estimated that over 40% of food is wasted annually, while one in six individuals is classified as food insecure (Gunders 2012). This equates to approximately 160 billion pounds and $165 billion every year in uneaten foodstuffs, and an extra $218 billion when the processing, transportation, and disposal costs are included (Harvard Food Law and Policy Clinic et al. 2016). In addition to the economic toll, this waste accounts for a large proportion of methane emissions from landfills; organic matter in landfills accounts for up to 16% of U.S. methane emissions (Gunders 2012), excluding methane produced from earlier steps in the food chain.

While a certain level of loss is expected, as with any commercial scale operation, losses experienced during food production are much higher than other industries. In industrialized countries like the U.S., over 40% of losses come at the consumer and retail level alone; of these losses, approximately 56% come from residences (EPA 2014). Consumer loss in industrialized countries has been estimated to be as high as 222 million tonnes annually, which is almost the entire net production of sub-Saharan Africa (230 million tonnes) (FAO 2018). Furthermore, losses differ by retail category; approximately 52% of fruits and vegetables and 38% of grain products are lost (FAO 2011; Gunders 2012), both of which would make ideal agriculture feeds if diverted to agriculture instead of landfills.

Initiatives to reduce or reuse these wastes include consumer education, policy or legislation to mandate food conservation efforts, and diversion of these materials to agriculture. Generally, these programs aim to marry sustainable infrastructure with convenience, incentives, and mandates to ensure consumer participation (CSWD 2018). By implementing these programs as something familiar to consumers, such as curbside pickup, consumer participation can be achieved at higher rates than voluntary enrollment alone. Additionally, food scrap collection services for the commercial sector are often less expensive than traditional hauling fees. To date, several pilot programs have shown significant savings and benefits to food scrap collection. For example, a partnership between the Rutgers University dining facility and nearby Pinter Farms saved over $100,000 in hauling fees, a 50% reduction in feeds paid to divert these materials to landfills (EPA and Rutgers University 2015). Additionally, the MGM Grand Buffet in Las Vegas was able to increase their food waste recovery by over 10,000 tons in just 5

years and save over $6,000 a month in partnerships with RC Farms and A1-Organics (Wright et al. 2015).

While these efforts are showing great promise, most of them are currently based in voluntary enrollment. Due to the magnitude of the problem, legislation is being introduced in many areas. By mandating these critical stop-gap measures, the billions of tonnes of food wastes can be diverted from landfills and used as a sustainable source of energy and fertilizer.

1.2.1. Legislation

In response to growing concerns over food waste, several states and cities have implemented legislation that mitigate these issues. To date, 13 states and 5 cities have passed legislation concerning food waste recovery: Vermont, Massachusetts, Connecticut, Rhode Island, and California along with New York City, Minneapolis, Seattle, Boulder, and Austin are leading the way to incentivize consumer food waste reduction and recovery. Varied in scope, these have a common goal to reduce co-mingled food in landfills and center around the food recovery hierarchy (Figure 1.1). The most comprehensive mandate at this time is Act 148 of Vermont, deemed the Universal Recycling Law, which will phase in universal bans on organic food wastes and is one of the only programs extending into residences.

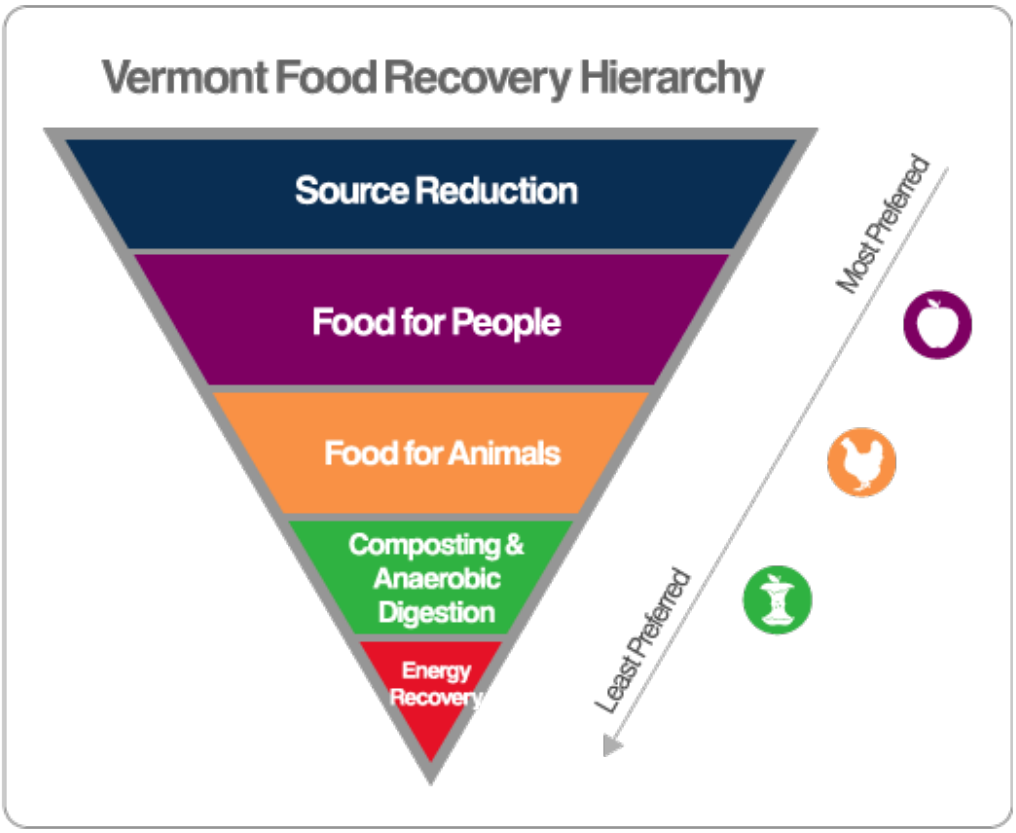


Figure 1.1 Act 148 Food Recovery Hierarchy (from the Vermont Agency of Natural Resources, 2015) demonstrating the fate and direction of food waste recovery efforts

Programs such as Act 148 will introduce and mandate food waste recovery at both the commercial and household level. Starting with large producers (over 104 tons/year) in 2014, by 2020 anyone producing food residuals in Vermont will be required to collect and divert these materials (State of Vermont 2012). Similar programs in other states typically involve only the largest producers, such as Massachusetts where only commercial facilities producing at least one ton of material per week are covered by the regulation (MassDEP 2018).

While no specific food recovery mandates have been implemented at the federal level, the EPA has issued a voluntary Food Recovery Challenge and produced guidelines for these programs through their "Food Recovery Hierarchy" (available at epa.gov/sustainable-management-food/food-recovery hierarchy). Goals of this challenge include an assessment of current practices, setting goals for food waste reduction, and a commitment to monitoring progress across areas of prevention, donation, and composting. The main targets of these programs include grocery stores, colleges and universities, and large sports/entertainment venues. In 2011, the EPA had enrolled 77 participants; by 2016 this number had grown to over 950 (EPA 2018). These resources also provide information about the current legal standards and implementation to guide new participants. Existing federal restrictions on how food wastes may be used, including the Swine Health Protection Act (SHPA), Ruminant Feed Ban Rule, and Food Safety Modernization Act (FSMA), are important to consider when participants are identifying partners and their ideal waste streams. At the state level, only the swine industry is widely regulated (in 48 states and Puerto Rico), while diversion to poultry production is only regulated in 13 states, ranging from strict prohibition (Georgia, Illinois, Iowa, Kansas, Michigan, and New Jersey) to requirements for licenses, heat-treatment, or feeding in only backyard operations (California, Minnesota, Nebraska, Nevada, New York, Pennsylvania, and Puerto Rico). Notably, Massachusetts requires heat-treatment for all materials, but this definition of "garbage" only extends to meat products and requirements for vegetable wastes is ill-defined (Harvard Food Law and Policy Clinic et al. 2016). As restrictions and requirements for additional processing such as heat

treatment are less widespread, the poultry industry is an ideal target for widespread food waste diversion.

1.2.2. Food Scraps in Agriculture & the Reemergence of "Garbage Feeding"

The use of food wastes as animal feed on a smaller scale has long been a part of the American and global agricultural systems. Dating back to the earliest agrarian societies and in modern times of resource conservation such as World War II (Gilbert 2017), it was common practice for family farmers to save household leftovers for their animals. However, these practices have been declining since the 1980s when outbreaks of diseases such as bovine spongiform encephalopathy (BSE) or foot-and-mouth disease (FMDV) were linked to animal feed and increased restrictions were put in place (Harvard Food Law and Policy Clinic et al. 2016).

Recently, both economic and environmental concerns have reinvigorated these so-called "garbage feeding" practices among many small farmers. Feed represents a significant portion of production costs in every industry; in poultry alone feed can account for up to 70% of production costs and 30% of retail egg prices (Gilbert 2017). In addition to a reduction in overhead costs, collection of food scraps often provides a direct source of income to farmers through tipping fees paid by food waste haulers (Composting Association of Vermont 2018) or additional income if the farmer acts as the hauler themselves.

Beyond the economic incentives, foraging from food scraps is a more natural way for poultry to feed. Ancestors of the modern chicken, Red Jungle Fowl, derived much of their diet through the decomposer system and allowing industrial poultry to feed this way

may be an additional animal welfare boost as well as an ecological one (Gilbert 2017). In the wild, fowl species can spend up to 61% of their time foraging; this behavior carries over into the domestic chicken, as they will continue foraging behaviors even when adequate feed is presented to them (Jacob 2015). Allowing poultry to feed from food wastes is also beneficial to water intake, as issues with dry feed clumping may be less likely to occur.

Poultry production presents an excellent solution to the dilemma of where to divert food scraps for agricultural use; with fewer restrictions on feeding and a natural willingness of poultry to consume their food this way, instituting food waste feeding as a management practice is ideal. These producers can kill two birds with one stone, gaining a source of feed for their animals and substrate for compost with whatever is left. Current food waste management programs include aerobic composting, anaerobic digestion, or direct feeding of scraps where applicable. For the purpose of this work, aerobic composting will be the focus as the infrastructure requirements are much lower than anaerobic digestion facilities and it is more likely to be used by small-scale farming operations. Composting at the farm level is not only an economic boost for farmers, but an infrastructural necessity. Of the 273 food waste composting facilities in the U.S., only 71 currently accept residential wastes; in New England, this number shrinks to 8 with a mere total of 16 facilities at the commercial or municipal level (Levis et al. 2010).

1.2.3. Food Waste Composting

In addition to the use of the food waste materials as animal feed, these organic wastes are destined to become substrates for composting operations. Through this process,

complex and co-mingled organic materials are broken down into relatively homogenous substances that can be used as soil amendments and fertilizers (Li et al. 2013; Cerda et al. 2018). As the current amount of food losses are much greater than can be reasonably absorbed as animal feed under current guidelines and production, a large portion of diverted food wastes are expected to be used for composting.

There are various compost management styles that can impact the characteristics of the finished compost. Popular approaches include windrow, in-vessel systems, tunnels, aerated static piles (ASP), or the Gore Cover system (Levis et al. 2010). However, due to the high moisture content and heterogeneity of food residuals, special considerations must be made, such as odor or contaminant removal. Previous work has found that of the common techniques, windrow systems are favorable for pathogen removal due to the higher temperatures and increased processing time (Cekmecelioglu et al. 2005). Various environmental factors, including temperature, pH, carbon/nitrogen (C/N) ratio, particle size, aeration rate, or nutrient content can also be adapted to improve the quality of the final product (Li et al. 2013). Finally, different materials added for "bulking" can affect the microbial activity and community, as well as reduce odor and improve ease of handling (Guidoni et al. 2018).

In addition to typical windrow approaches, some facilities have added vermicomposting to their processing scheme. Vermicomposting refers to the mesophilic process of using decomposer species, such as earthworms or housefly larva, to further stabilize organic residues in waste materials (Anastasi et al. 2005; Wang et al. 2015). This process results in a highly-nutrient rich product. In addition to be a value-added product from the food composting process, vermiculture has been shown to have positive impacts

on microbial content and attenuation of ARGs. Neher et al. (2013), reported that vermicompost samples had increased microbial diversity that may be favorable for plant growth and biological control when compared to windrow and aerated static pile counterparts. More recently, housefly larvae were successfully used to reduce ARGs and key integrase gene *intl1* in swine manures in only 6 days, compared to 2-3 months to achieve similar results by traditional composting alone (Wang et al. 2015). In a worm-based vermicomposting system, GFP labeled *E. coli* was cleared to below EPA compost sanitation guidelines in 18-21 days, compared to 51 days without earthworms. This mitigation was proposed to be the result of antagonistic effects of dominant community members rather than the heating associated with traditional thermophilic composting (Hénault-Ethier et al. 2016).

Regardless of management practice, food waste composting presents several challenges. In addition to being highly variable in composition, food wastes are high in moisture, organic to ash ratio, and frequently contain other waste materials such as plastics (Cerda et al. 2018). Moisture content can range from 74-90% and C/N ratio as much as 14.7-36.4 as shown by a global survey (Thi et al. 2015), making best-practices hard to define. Other common measures of compost quality and effectiveness, including temperature, oxygen content, moisture, particle size, or compaction (Li et al. 2013), can be difficult to consistently maintain across batches of food waste. These factors make additional research in this field a necessity, especially given the lack of data on the fate of ARB or ARGs in these materials.

1.2.4. Environmental Routes of Resistance Transfer

In addition to clinical or nosocomial transfer of antimicrobial resistance, due to issues such as patient non-compliance and improper prescribing practices (Berglund 2015), environmental transmission of both ARB and ARGs has been well documented (Pruden et al. 2013; Bengtsson-Palme 2017; Hiltunen et al. 2017). In fact, evidence suggests that the environment is not only a reservoir for antimicrobial resistance, but the source itself (Davies and Davies 2010; O'Toole 2014; Berglund 2015). Resistance genes are increasingly thought of as a separate class of contaminant and risk factor due to their ability to undergo gene transfer after bacterial death and presence in a variety of environmental contexts (Pruden et al. 2006; Liss et al. 2016). Current examples of ARG surveillance include cattle and swine manures (Zhu et al. 2013; Udikovic-Kolic et al. 2014; Wichmann et al. 2014; Ross and Topp 2015; Noyes et al. 2016; Qian et al. 2016), municipal solid wastes (Ross and Topp 2015; Ju et al. 2016), wastewater effluents (Pruden et al. 2013), and even paper currency (Jalali et al. 2015). Generally, these efforts have found that manure treatments without composting can lead to significant increases in ARGs in soils (Zhu et al. 2013; Udikovic-Kolic et al. 2014; Ross and Topp 2015), but thermophilic composting or anaerobic digestion can reduce the overall load of resistance in these materials (Qian et al. 2016; Liao et al. 2017).

Monitoring of food wastes and residuals is limited compared to these sources. While speculation at this time, these materials may carry an increased risk due to their intrinsic ability to act as a fomite, as well as increased human contact that may introduce pathogenic species or additional ARGs. Researchers have demonstrated the presence of ARBs or ARGs in ready-to-eat foods, meats and other animal products, and a variety of produce (Bezanson et al. 2008; Silveira-Filho et al. 2014; Sultana et al. 2014; Doyle 2015;

Kevenk and Gulel 2016). However, due to the many avenues of transfer defining a source for these ARGs and ARB is difficult; contamination may occur due to improper handling of meats, use of antimicrobials during production, or simply from the surrounding environment and soils that produce is grown in (CDC, 2017). As a result, controlling the presence of ARGs in food products would be a Sisyphean task. Instead, limiting the transfer and selection of multidrug resistant (MDR) or clinically relevant genes among food products and wastes is the more prudent food safety measure (Godziszewska et al., 2016) these materials is the most relevant path in terms of food safety.

In addition to the innate nature of food scraps as a vehicle for transfer, human activities may increase the risk associated with these products. Use of antimicrobials or disinfectants that select for resistance within the consumer household may increase the prevalence of resistant organisms on food wastes. Studies have linked use of household disinfectants, such as triclosan, to selection of antibiotic resistance (Webber et al. 2017). In fact, triclosan is a listed component of several cutting boards, kitchen utensils, dish soaps, and an incredible number of other household products (US Department of Health and Human Services 2018). Again, while not specifically tested at this time, the potential for selection in the household or consumer setting suggests that both industrial and post-consumer food waste may be a significant source of antibiotic resistant bacteria and genes onto the farm and should be quantified in order to create proper management plans.

As shown in Figure 1.2, there are several known routes of transfer between the environment, agricultural, and human activities. In a recent review, Verraes et al. (2013) summarized potential transferred events in the food chain into three routes: selection of ARB due to antimicrobial use during production, presence of ARGs in bacteria added

during food processing (*i.e.*, starter cultures and bacteriophages), or contamination with environmental ARB/ARGs during production. Application of manures as field amendments (Udikovic-Kolic et al. 2014; Peng et al. 2017), runoff and wastewater effluents (Zhang et al. 2016), and overuse of antimicrobials in agriculture (Economou and Gousia 2015; Thanner et al. 2016) have been widely implicated as routes of transfer and sources of resistance within these categories. Additionally, transmission by direct contact of humans and animals (Marshall and Levy 2011), drinking water (Xi et al. 2009; Bergeron et al. 2015; Xu et al. 2016), and various food products (Marti et al. 2013; Chajęcka-Wierzchowska et al. 2016; Yang et al. 2016; Baloch et al. 2017; Sanchez 2018) have been shown to contain ARB or ARGs. This widespread occurrence in ready-to-eat products is particularly concerning, as unconsumed portions are likely to end up as food wastes, yet surveillance of these materials stops at the point of consumer purchase. One could argue that post-consumer food wastes presents the opportunity for not only co-mingling of antibiotic resistance from all of these sources, but concentration as well. By the time food products have reached the stage of human consumables, they have experienced each of the events highlighted by Verraes et al. (2013), and as post-consumer waste and residuals they are comingled at a single location for processing and subsequent dissemination to agricultural production.

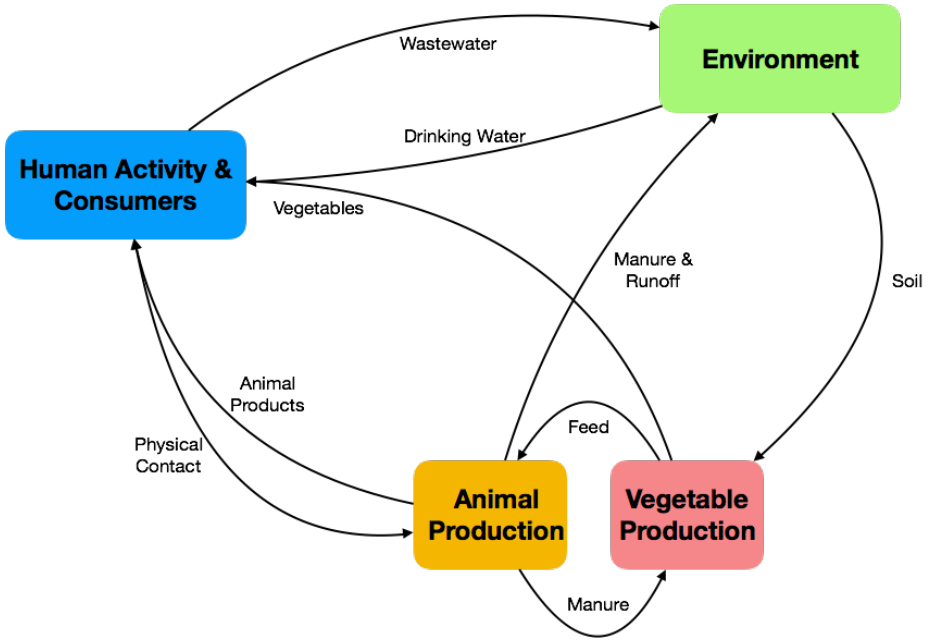


Figure 1.2 Currently characterized routes of ARB or ARG transfer. Arrows indicate direction of transfer.

There is a gap in knowledge on this potential link between human-generated food wastes being returned to the farm environment; the spread from 'table-to-farm' may be a critical point of entry for novel ARGs or ARB into the farm environment, where they may then be amplified and make their way to the community at large, perpetuating the cycle. Preliminary research into the vertical integration of pathogenic species such as *Salmonella enteritis* from food scraps into eggs has been performed on three farms in Vermont so far, with no findings of elevated pathogenic load (Composting Association of Vermont 2018). However, prior to this work, there has been no investigation into the fate of ARGs or associated pathogenic bacterial species. As food waste feeding becomes more popular as a

management practice, additional surveillance and risk assessment of antimicrobial resistance transfer must be conducted.

Risk assessments have been performed for similar materials, such as municipal solid waste composting or animal manure composting (Domingo and Nadal 2009; Thanner et al. 2016). These reviews have identified associated human health risks, such as emitted toxins, organic dusts and other bioaerosols, fungal exposure (Domingo and Nadal 2009) with municipal wastes, and often consider manure as a "hot spot" for resistance due to the selection of bacteria carrying ARGs on mobile genetic elements (Thanner et al. 2016). Food scraps may act as a similar risky material as they contain an abundance of fungal species that could be aerosolized and microorganisms undergo the same co-mingling and period of stress associated with increased risks in manures and solid wastes. There are several mechanisms of potential spread once food wastes make their way onto the farm (Figure 1.3) including plasmids, phages, transposons, or other mobile genetic elements (MGEs) that may survive the composting process even if the microorganisms themselves do not.

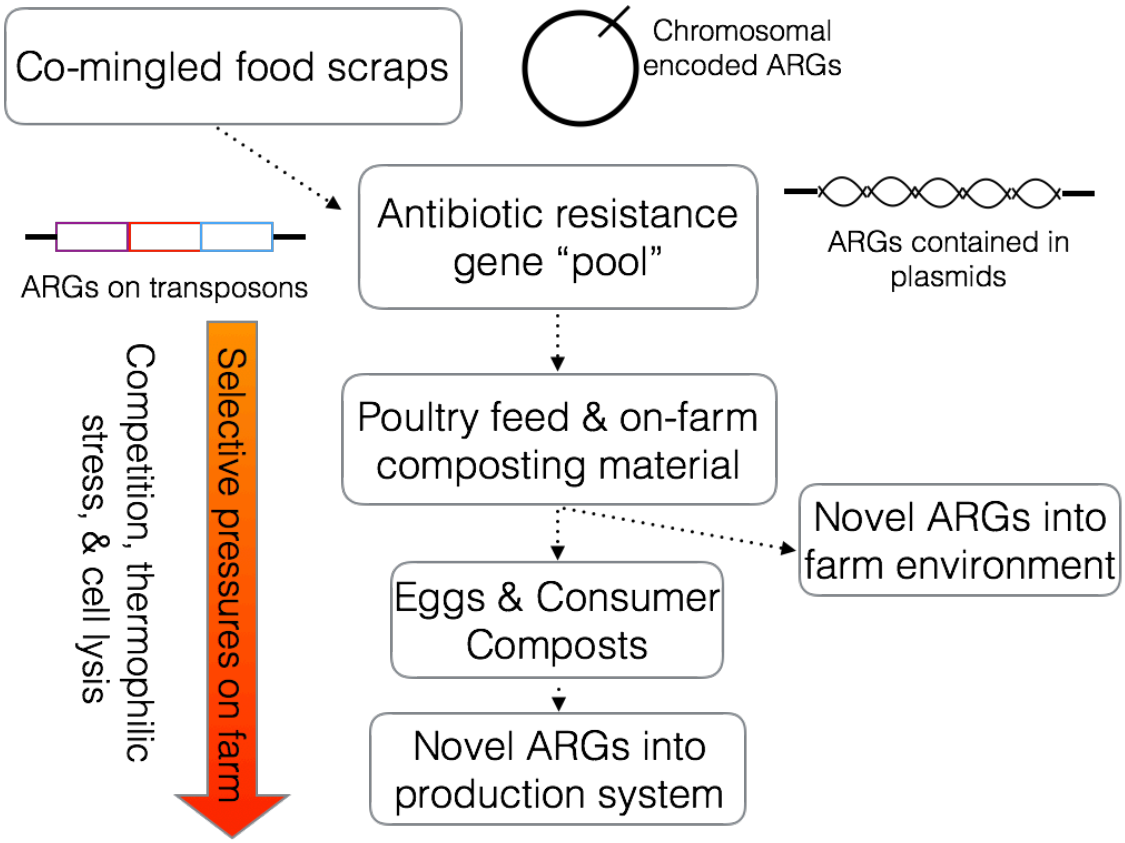


Figure 1.3 Avenues of ARG spread or selection upon introduction to the farm setting. ARGs can be carried on several types of genetic elements, and selection pressures during the food scrap composting process are poorly described.

1.3 Global Burden of Antimicrobial Resistance

The looming threat of multi-drug resistant microorganisms has made its way to the forefront of global priorities, concentrating efforts across disciplines in an attempt to limit the spread and dissemination of these pathogens across environments. Determining the true cost of antibiotic resistance has proven difficult due to the complex nature of the issue; a recent review conducted by RAND Europe has focused on the economic impacts, specifically those related to increased mortality that will decrease the global workforce and

increased morbidity that will reduce the productivity of remaining workers. Of the scenarios tested, their estimates concluded that by 2050 the global workforce will be reduced between by 11 to 444 million, leading to a decrease in global Gross Domestic Product (GDP) by 0.06-3.1%. (The World Bank 2016).

In addition to economic impacts, our ability to treat clinical infections is decreasing as multidrug resistance spread is increasing. Production of new antimicrobials is slowing for two major reasons. Scientifically, discovery of novel mechanisms, including those to which bacteria cannot readily develop resistance, is limited. Fiscally, antibiotics have an extremely low return on investment compared to other pharmaceuticals; companies simply aren't earning enough to justify the millions spent on drug development (Braine et al. 2011).

ARB are responsible for the infections of over 2 million people and 23,000 deaths each year in the U.S. alone. Beyond the toll on human health, the primary economic cost of these infections hovers around $20 billion a year (U.S. Department of Health and Human Services 2013). In addition to acute illness, foodborne diseases caused by pathogenic or resistant species compound this issue. The CDC estimates that each year 48 million people get sick in the U.S., 128,000 are hospitalized, and 3,000 die of foodborne diseases; 1 in 5 of these (*i.e.*, 9.6 million people) are infected with antibiotic resistant bacteria (CDC 2016). Of these foodborne illnesses, 46% of exposures are attributed to produce, and 29% of deaths are attributed to meat (CDC 2013).

Globally, the main impacts of antimicrobial resistance are falling GDPs and an increase in poverty, especially in low-income countries where an estimated 28.3 million people would be pushed into extreme poverty by 2050 (The World Bank 2016). Estimates

of secondary costs, *i.e.*, those beyond ambulatory antibiotic prescription, including hospitalization, outpatient and second-line prescribing, and antibiotic stewardship can add as much as $4.4 billion to current U.S. estimates (Michaelidis et al. 2016). These projections demonstrate the reach of antimicrobial resistance goes far beyond the hospital bed. In addition to reducing the clinical cases of antimicrobial resistant infections, efforts to reduce the spread of resistance in the environment and food systems plays an important role in global efforts to curb this crisis.

1.3.1. U.S. Action Plans and Priority Areas

According to the CDC, there are four core areas requiring "aggressive action" to fight antimicrobial resistant bacteria. These include i) preventing infections and the spread of resistance, ii) tracking resistant bacteria, iii) improving the use of today's antibiotics, and iv) promoting the development of new antibiotics and developing new diagnostic tests for resistant bacteria (CDC 2018). The third aim is already underway in agricultural production in many areas with the inclusion of judicious use of antimicrobials in food-producing animals. In effect since 2017, U.S. policies mandate the veterinary supervision of antimicrobials and will no longer allow for the purchase of these substances over-the-counter (FDA 2018). The other aims are intertwined in rigorous surveillance programs, as the tracking, diagnostics, and mitigation all require significant knowledge in order to be most effective. The U.S. National Action plan also calls for the strengthening of One-Health surveillance efforts to combat resistance (The White House 2015), acknowledging the significant role of transmission between the environment, humans, and animals.

Specific objectives for curtailing the spread of antimicrobial resistance, including both ARB and ARGs, through the environment and food production chain are included in action plans from groups including the USDA (USDA 2014), the Environment Agency in Europe (Singer et al. 2016), and WHO (WHO 2015). The WHO and USDA guidelines in particular recognize the role of the animal production environment, both for its selective pressures and risk of dissemination into the human population. The ubiquity of ARGs within the natural environment (Szekeres et al. 2017; Pruden et al. 2006; Berglund 2015; Pal et al. 2016) poses an increased risk for horizontal gene transfer, as heavy metals (Singer et al. 2016) and plant derived chemicals (Friedman 2015) present in soils can provide a selective pressure even in the absence of antimicrobials or residues. Additionally, co-selection for ARGs can occur via co-resistance or cross-resistance, meaning a specific gene is either transferred due to the presence of a linked gene or confers resistance to multiple chemicals that may be present in the environment (Singer et al. 2016). Despite these calls to action and acknowledgement of increased risk, there is a large knowledge gap where food waste composting and diversion are concerned.

1.4. Mechanisms of Horizontal Gene Transfer

Bacteria can acquire antibiotic resistance by a number of molecular mechanisms. While many bacteria are intrinsically resistant to certain antibiotics due to chromosomally encoded genes, it is currently believed that a significant portion of bacterial genomes are composed of genes acquired by horizontal gene transfer (HGT) (Nakamura et al. 2004). Through these mechanisms, genes found on mobile genetic elements can be transferred to both closely related and divergent species of bacteria. Induction of HGT, regardless of mechanism, can be triggered by selective pressure, including presence of antibiotics, heavy

metals, or other environmental stressors. The idea that composite composts, such as manures, municipal wastes, and sewage, might induce horizontal gene transfer is hardly new; Riber et al. (2014), Xiong et al. (2015), Ross et al. (2015), and others have all tested similar hypotheses.

When transferred, genetic elements conferring significant fitness advantages are likely to become fixed in a population or environmental niche, even in non-pathogenic species that are able to act as a reservoir of resistance. Relevant to this work is the ability of bacteria to integrate mobile genetic elements from their "deceased" companions. During the composting cycle, microorganisms incapable of surviving the thermophilic phase are subject to cell lysis. When this happens, extracellular DNA or mobile genetic elements, in this case ARGs, can be assimilated into the remaining microbiome (Pruden et al. 2006; Jakubovics et al. 2013; Vorkapic et al. 2016); if these genes allow for an advantage they may become fixed within these compost materials, even on organic farms or those not actively using antimicrobials. As a result, researchers are beginning to recognize that not only are pathogenic species of concern when assessing the risk of materials within the food system, but the fate of mobile genetic elements containing ARGs or other functional genes related to virulence must be evaluated.

1.4.1. Transformation

The first mechanism of gene transfer to be discovered (Griffith 1928), transformation involves the direct acquisition of genetic material from the surrounding environment. This is generally thought of as "naked DNA" from the surrounding environment, either due to cell lysis or cell death, and requires recipient cells to be in a

state of competence. A tightly regulated physiological state, competence is often activated in response to specific growth conditions, cell-cell signaling known as quorum sensing, or starvation, and varies greatly across bacterial species (Thomas and Nielsen 2005). Some bacterial cells are naturally competent, including *Campylobacter spp., Bacillus subtilis* and *Streptococcus spp*., and may undergo transformation at any time (Johnsborg et al. 2007). This process may also involve integrons and plasmids for additional stabilization and genome integration and is thought to play a role in the transfer of genetic material between bacterial species of distant relation (Thomas and Nielsen 2005).

Due to the nature of DNA, (*e.g.*, "naked DNA" is susceptible to nuclease activity, as well as physical and chemical degradation), transfer by this mechanism is less likely to occur and less detectable in most settings (Verraes et al. 2013). While not of greatest concern when discussing clinical acquisition of resistance, the potential for natural transformation to occur upon cellular death during composting must be mentioned. Bacterial cells are known to undergo lysis during the stress of composting, and previous work has shown that 1 µg of extracellular DNA per gram of soil can be isolated (Ogram et al. 1987). It may take a perfect storm to lead to significant ARG transfer by natural transformation during food waste composting, but in the world of bacterial transformation, nothing can be ruled out. For example, transformation events have been documented in the sausage making process due to protection by biofilms (Straub et al. 2016).

1.4.2. Transduction

Transduction involves the transfer of genetic material between microbial cells via intermediaries known as bacteriophages, or now commonly referred to as simply phage.

Initially described in *Salmonella* (Zinder and Lederberg 1952), transduction has now been observed in a wide variety of bacterial species. Once a bacteriophage is attached to the host bacterial cell and injects its genetic material, it will either form a separate replication element or integrate into the host genome (Verraes et al. 2013). In either method, bacteriophages utilize the host machinery to enable replication of phage particles.

This mode of transfer has been widely identified as a common method of ARG transfer, particularly as the popularity of "viromics" has grown in tandem with microbiome studies. For example, the phage transferred *qacB* among *Staphylococcus aureus* has been documented (Nakaminami et al. 2007), as well as tetracycline and gentamicin resistance among enterococci (Fard et al. 2011) or antimicrobial resistance plasmids in methicillin resistance *Staphylococcus aureus* (Varga et al. 2012). However, previous work may have overestimated the true rate of ARG transfer by transduction due to false positives introduced by sequence similarity-based analyses, and the true prevalence of ARG encoded by phages is much lower (Enault et al. 2016).

1.4.3. Conjugation

Of these mechanisms, conjugation is thought to be the most "risky" in the spread of clinically relevant resistance genes (Mathur and Singh 2005; von Wintersdorff et al. 2016), as the physical contact of cells protects DNA from potential damage in complex environments such as soils and composts and often involves relatively small plasmids that can be easily spread without significant fitness costs to the host. Conjugative transfer has been described as far back as 1946 by the team of Joshua Lederberg and Edward Tatum (Freeman 2018), and can occur with a variety of cell-cell junctions, including pili in gram-

negative species or pheromones in gram-positive (Hirt et al. 2002). This method requires the physical contact of a donor and recipient cell and is often likened to a type of bacterial "sex". The 'male' donor cell transfers genetic material via an encoded apparatus, most commonly a pilus, which is accepted by the 'female' recipient cell. Additionally, conjugative transfer is more efficient at entering host cells compared to transformation, and has a broader host range than transduction (von Wintersdorff et al. 2016).

Conjugation can include both plasmids and transposons, classified as Integrative Conjugative Elements (ICE) or Integrative Mobilizable Elements (IME) that can also contain genomic pathogenicity islands (Verraes et al. 2013). The combination of these traits allows HGE events to occur across a broad range of bacterial species and environmental conditions. Transfer of ARGs via conjugation has been observed in a wide range of hosts and environments, including Tn916 that can transmit DNA in over 50 species or A^R-P that has been seen in soils, urinary tracts, sewage, and marine environments (Davison 1999). Other types of conjugative elements include cassettes, such as the *Staphylococcal cassette chromosome* (SCC) that can transfer resistance genes such as *mecA* among *Staphylococcal* species (Haaber et al. 2017). In fact, Haaber et al. (2017), documents over 45 cases of ARG transfer by conjugation in *S. aureus* alone.

1.4.3. Gene Transfer Agents

More recently, a fourth mechanism of HGT has been described. Gene transfer agents (GTA) are phage-like elements that are found in many prokaryotes. Unlike the other three mechanisms, they contain random segments of a cell's genome and can be thought of as particles rather than complete genetic elements. Most GTA will not contain functional

coding elements, and instead may act as a last resort of preserving genetic material upon cell lysis (Lang et al. 2012). Transfer of DNA via this mechanism appears to be within strict host ranges, where individual bacteria within a colony or population sacrifice themselves and release GTA via cell lysis (Québatte et al. 2017). Transfer of GTA genes has been observed in marine bacterioplankton and may be responsible for genomic plasticity in environments where more common HGT mechanisms are not available (Biers et al. 2008). While the likelihood of a complete antibiotic resistance gene being transferred by this route is low compared to the traditional mechanisms of HGE, transfer of resistance markers has been observed in *R. capsultas* and *B. hyodesenteriae*, with transfer of the *B. hyodesenteriae* GTA VSH-1 being induced by antibiotics (von Wintersdorff et al. 2016). As such, this mechanism of transfer cannot be ruled in the complex conversation surrounding environmental reservoirs of resistance.

1.5. Molecular Methods of ARG Detection and Sequencing

There are many strategies for the detection, surveillance, and profiling of ARGs and microbial communities. Historically the focus has been on pathogenic species by isolating pure cultures and assessing resistance using culture-dependent assays and defined clinical breakpoints such as EUCAST. However, these methods are labor intensive, low throughput, and limited to species that can be grown efficiently in culture. As a result, culture-independent techniques for ARG detection are becoming more popular, including quantitative PCR (qPCR), microarrays, and recently shotgun metagenomic sequencing. As implementation of these methodologies is increasing, our ability to monitor the spread of

antimicrobial resistance throughout the environment and food systems becomes more robust.

1.5.1. qPCR

An example of a targeted/PCR-based approach, quantitative PCR (qPCR) and quantitative real-time PCR (qRT-PCR) allow the characterization of specific genes from a wide variety of DNA samples. These techniques use either fluorescent probes or dyes that bind to the target sequence. The advantage over traditional PCR is that gene copy number is detected during every cycle of the reaction, allowing for the direct quantification of ARGs when compared to a standard curve (Luby et al. 2016). In addition to singular reactions, qPCR arrays for ARGs are now available. These arrays allow for the quantification of large numbers of ARGs or other targeted genes at once. Commercial kits for this purpose are available, such as the Qiagen Antibiotic Resistance Genes Microbial DNA qPCR array or the Wafergen Bio-systems SmartChip Real-Time PCR. Previous examples using these approaches include surveillance of aquaculture, swine production, and municipal wastewaters (Volkmann et al. 2004; Zhu et al. 2013; Muziasari et al. 2016).

The main drawbacks of this approach are the necessity for prior sequence knowledge, total number of genes that can be screened in a reaction or assay, and detection limit (Smith and Osborn 2009). In order to design probes for qPCR, the target gene sequences must be known making novel gene or variant discovery impossible. While qPCR arrays represent significant progress towards increasing throughput, these are still limited to genes on the order of hundreds and may be a limitation in large-scale surveillance efforts. Finally, limits of detection are strictly related to DNA input volume, which is typically on

the order of nanograms for qPCR arrays (Luby et al. 2016). Additionally, like the other targeted tools discussed in this section, quantification of only ARGs or other marker genes is accomplished by this method and additional tools are required for characterization of the associated microbiome.

1.5.2. DNA Microarray

Moving towards increased automation, microarray chips allow for the detection of thousands pre-selected genes in a single hybridization assay (Bumgarner 2013). Microarray chips leverage knowledge of gene sequences to create oligonucleotide probes which are adhered to modified microscopic slides. This allows for the creation of custom arrays and rapid detection of known sequences from a variety of extracted DNA. Similar to qPCR arrays, these have the added benefits of sheer abundance of probes. In contrast to qPCR where researchers are typically querying for a more limited number of specifically chosen genes, microarray chips can be useful when the goal is quantification of a greater number of targets and can be more easily automated.

Microarray analysis has been successfully used to identify ARGs in a variety of studies. For example, Lu et al. (2014) used a microarray chip covering 369 resistance types to identify a link between the age of the human host and resistance gene diversity. This tool has also been applied to the detection of ARGs and virulence factor genes in tandem, allowing for increased throughput of clinically relevant species (Walsh et al. 2010). Commercially available options, such as ArrayTubes, are capable of detecting ARGs from both complex samples, such as milk, and individual isolates in order to improve surveillance in agricultural systems (Perreten et al. 2005).

While microarrays are a flexible and rapid tool for ARG detection, they come with several caveats. Most obvious is the physical limitation of the chip itself; only sequences placed onto the array can be queried and information pertaining to novel sequences will be missed. Additionally, results can be difficult to interpret, as non-functional or non-expressed genes may be detected (Frye et al. 2010). Finally, this method only provides information on the resistance genes themselves and must be complemented by additional tools to identify pathogenic species present or gene expression.

1.5.3. Shotgun Metagenomic Sequencing

Shotgun metagenomic sequencing is emerging as a comprehensive tool for the study of environmental systems. Combining the advantages of culture-independence with the ability to characterize the total composition of each sample in one sequencing effort has afforded greater insight into the dynamics and diversity of antimicrobial resistance transfer. Shotgun metagenomics refers to the lack of target for sequencing; in this approach, the entirety of extracted DNA is fragmented ("shotgunned") and subsequently sequenced (Sharpton 2014). With this single technique, researchers can identify sample biodiversity without the primer bias and limitations of amplicon sequencing and characterize resistance and functional genes without *a priori* expectations. One of the greatest advantages of shotgun metagenomics is its ability to not only identify community composition without bias, including bacterial, fungi, and protists, but also characterize functional genes present to begin to answer what these organisms might be doing.

Prior studies have successfully utilized shotgun metagenomic sequencing as a screening tool for antibiotic resistance genes in a variety of settings. It has been

successfully used to characterize resistomes of lakes (Bengtsson-Palme et al. 2014), paper money (Jalali et al. 2015), manures and agricultural soils (Durso et al. 2012; Wichmann et al. 2014), and hospital effluents (Rowe et al. 2016). In these instances, researchers were able to not only identify ARGs present, but also assessed the potential mechanisms of gene transfer and microbial context of each sample type. Furthermore, the resolution of this method can reach the level of bacterial strain with sufficient sequencing depth and does not introduce the same primer bias associated with PCR-based techniques (Shah et al. 2016; Yang et al. 2016b).

The flexibility and range of data produced makes shotgun sequencing an ideal technique for food safety and environmental surveillance. It has been successfully used to detect foodborne pathogens in various stages of beef production (Yang et al. 2016b) and Shiga-toxin producing *E.* coli from spinach (Leonard et al. 2015) While it lacks the exact quantitative abilities of qPCR, shotgun sequencing can identify putative novel genes, patterns of co-resistance, and genomic context of ARGs (Bengtsson-Palme et al. 2017).

However, it is much more data intensive and requires specific considerations in data analysis due to its complexity. Additional considerations must be given to sample preparation, sequencing depth, and sequence analysis that are not required of more targeted approaches. As all DNA is sequenced, any contamination or bias introduced during sample collection, processing, and DNA extraction will be carried forward. Reagent contamination and natural variation in GC content (Dohm et al. 2008; Knauth et al. 2013; McCarthy et al. 2015) can present issues at the DNA extraction phase, while sample storage can impact results even earlier (Choo et al. 2015; McCarthy et al. 2015). After sequencing, users are presented with an array of choices for analysis and ARG annotation; at least 19 databases

exist for ARG annotation alone, each relying on different methods of classification (Xavier et al. 2016).

In addition to these considerations, metagenomics cannot determine expression of any resistance genes found. As with other DNA-based molecular methods, only sequence of putative ARGs can be detected which may simply be non-functional copies or incomplete relics. Additional tools are required to assess expression and functional resistance, which is a staple of true public health analysis and epidemiological modeling. To combat this, some researchers have used shotgun metagenomics as a tool for more targeted qPCR or in tandem with popular culture-based techniques such as MIC determination and cfu counting to determine the functionally resistance and effective population within a sample (Munk et al. 2017).

1.5.4. Functional Metagenomics

Finally, a melding of traditional microbiology and advanced next generation sequencing has led to a class of techniques deemed functional metagenomic sequencing. A disadvantage of the culture-independent approaches described above is they only identify the presence of the genetic elements and they do not demonstrate functional antibiotic resistance. This can be remedied using a function metagenomic approach. This methodology involves the fragmentation and insertion of DNA into plasmids, transformation into competent laboratory strains of bacteria, and plating on selective media of choice (Luby et al. 2016; Boolchandani et al. 2017). Surviving colonies are subsequently sequenced and ARGs can be annotated. This approach has been used to assess functional resistance in food products such as cheese (Devirgiliis et al. 2014), manures and gut

microbiome of chickens (Zhou et al. 2012), and for the discovery novel antibiotic functions (Pehrsson et al. 2013). It has advantages even when compared to culture-dependent approaches, as use of indicator species allows detection of ARGs from species not readily grown in culture and allows for query against massive clone libraries (Boolchandani et al. 2017).

This technique does have limitations due to the associated labor and assay costs. Projects often involve thousands, if not millions, of clones that need to be screened against a suite of antibiotics. Compared to metagenomic sequencing alone this requires significant technical know-how as well as additional equipment for DNA fragmentation and ligation. Additionally, choice of the plasmid and bacterial host species is critical, as some may not be able to express all ARGs from environmental samples (Mullany 2014).

1.6. Bioinformatic Analysis Tools

The investigative power of metagenomics has led to its application in a variety of disciplines, and with it has risen the need for rapid, approachable, and reliable analysis tools. Characterization of shotgun sequences typically fall into four categories: alignment to references, composition or *k*-mer analysis, phylogenetics, or assembly (McIntyre et al. 2017). These tools offer solutions based on the problem of interest. For examining large community shifts one might forego a read-based alignment approach for the speed of a phylogenetic or marker gene based analysis; researchers requiring species level data and removal of false positives will likely choose tools that favor of improved classification and require greater computational power or time. In certain instances, combining several tools may be required for a robust analysis.

The breadth of algorithms and tools available for taxonomical analysis has prompted several evaluations of their speed, accuracy, and reproducibility (Vázquez-Castellanos et al. 2014; Lindgreen et al. 2016; Nayfach and Pollard 2016; McIntyre et al. 2017; Quince et al. 2017; Vollmers et al. 2017). These reviews aim to provide concrete evidence of algorithmic performance, typically utilizing controlled or mock communities. This research has shown that factors such as read coverage, choice of marker gene, sample complexity, and sequencing platform can all affect algorithmic performance. Recommendations for analysis method tend to be hypothesis specific. If metagenome assembly is the goal, longer reads from platforms such as MinION Nanpore or PacBio combined with high-quality assemblers like PhyloSift or CLARK are required; for researchers desiring relative abundance of many species, shorter reads from Illumina and high precision classifiers such as GOTTCHA or BlastMegan are more appropriate (McIntyre et al. 2017). If computational resources are scarce, cloud-based tools such as MG-RAST, One Codex, or CosmosID provide valuable alternatives.

In order to annotate antibiotic resistance genes, additional tools or databases are often required. Tools such as MG-RAST, One Codex, and CosmosID include this analysis in their pipelines, but specific ARG annotation tools can be added to any pipeline. Popular databases include CARD/ARDB (McArthur 2013), ARG-ANNOT (Gupta et al. 2014), MEGARes (Lakin et al. 2017), and ResFinder (Zankari et al. 2012). Much like taxonomic tools, these vary in their detection method and curation. For example, MEGARes is a hand-curated database targeted at population-level resistance profiling, rather than protein prediction or functional annotation of individual samples using CARD. Again, researchers must make a choice in tools based on individual hypotheses. Unfortunately, benchmarking

analysis like that of taxonomic methods is limited. Xavier at al. (2016) performed a minireview of four of the most popular tools, highlighting issues such as delayed curation, false positives, and nomenclature. Comprehensive analysis, benchmarking, and standardization of protocols will be necessary as shotgun sequencing for ARG surveillance becomes more popular.

1.6.1. Cloud-based Tools

Due to the sheer volume and complexity of data produced by shotgun sequencing, access to suitable computational tools can be a barrier to researchers or smaller clinical facilities. Frequently, the assembly and analysis of shotgun metagenomics data requires high-memory machines that may run for days (Thomas et al. 2012) and may not be something that every researcher has available to them. To address this, cloud-based solutions for bioinformatic analysis have become increasingly popular. These range in utility from simply off-loading the analysis to a remote server, such as Amazon's AWS or Galaxy, to fully-functional pipelines like MG-RAST, One Codex, CosmosID, and a growing market of competitors. Use of these tools allows for an efficient, reproducible, and scalable analysis that is more accessible to researchers than traditional command-line or cluster-based bioinformatics. Cost of these tools is either free at a basic level or typically in a "pay-as-you-go" model; rather than having to budget for the purchase of a new computer or flat annual fee of a university cluster, these platforms only charge for the time you use them (Amazon) or number of samples processed (CosmosID, One Codex). Services such as MG-RAST are even free, but depending on server loads may take several months to complete a job.

This project utilized CosmosID for analysis (CosmosID, Inc. Rockville, Maryland), a commercial bioinformatics software boasting over 150,000 curated genomes and quarterly updates to reference databases, a feat that cannot be replicated by many open-source solutions. In a recent review, this software ranked highest in identification accuracy even at the sub-species level (McIntyre et al. 2017). The ability to characterize samples at this resolution is critical to metagenomics research, as there is wide variation in phenotype across species, especially when considering virulence and antimicrobial resistance. Analysis is completed in as little as a few minutes, making it a streamlined tool for both clinical and ecological studies.

1.7. Conclusion and Aims

The need for surveillance and monitoring of antimicrobial resistance across the food system is more pressing than ever. Agricultural production is a significant reservoir of both ARB and ARGs (Thanner et al. 2016). We propose the food residuals and materials produced during food waste composting may be an emerging intermediary to transfer of resistance between the table and farm.

It has been shown the composting process mitigates many pathogenic species and leads to a reduction of ARGs in other materials (Wichmann et al. 2014; Wang et al. 2015), but this work has yet to be conducted in food waste composts. Food residuals represent a unique risk, as they are co-mingled from a wider variety of sources and are less consistent in composition between batches than traditional composts.

The limited knowledge of resistance in food residuals and composts has motivated the current study, where we seek to characterize resistance in a variety of inputs, compost

stages, and consumer products on an integrated poultry farm. The main aim of this work was the identification of ARGs and putative pathogens in these substrates and characterization of samples to elucidate potential transfer mechanisms. Further aims include demonstrating reproducible and accessible methodologies for the surveillance of these substrates, from sample processing and DNA extraction to shotgun metagenomic sequencing and analysis. These techniques are in line with national priority areas and action plans, demonstrating how novel diagnostics and tools can be used to improve surveillance and tracking of antimicrobial resistance in a variety of systems.

CHAPTER 2: Table to Farm

2.1. Abstract

Popularity of food waste diversion and composting practices, due to both mandate and accessibility, are a growing alternative to traditional waste disposal. An acceptable source of agricultural feed and composting material, these management practices divert methane-emitting food residuals from landfill and recapture nutrients that would otherwise be lost. However, risk associated with the transfer of antimicrobial resistant bacteria (ARB), antibiotic resistance genes (ARGs), or pathogens is not well characterized. Using shotgun metagenomic sequencing, ARGs were successfully identified across samples from an integrated poultry farm, as well as microbial content and associated virulence factors. A total of 495 distinct bacteria (at the species or sub-species level), 50 ARGs, and 54 virulence genes were found. Most prominent were ARGs related to aminoglycoside, tetracycline, and macrolide resistance, while most virulence genes were related to transposon or integron activity. Microbiome content was distinct between on-farm soils and off-farm collection sites, with a reduction in human pathogens throughout the composting process. Additionally, while most samples contained some level of resistance, only three resistance genes occurred in both on and off-farm samples and no MDR genes persisted once on the farm. Therefore, the risk of incorporating novel or multi-drug resistance from human sources appears to be minimal and the practice of utilizing human food scraps as feed for poultry and composting material may not present a significant risk for human or animal health. In addition to characterizing sample contents, Pearson correlation and co-inertia analysis was performed to identify any potential relationships between functional genes and microbial content. The most significant interaction appeared

to be between resistance and virulence genes ($P = 0.05$, RV = 0.67), indicating that ability to undergo gene transfer may be a better marker for ARG risk than presence of specific bacterial species. This work expands the knowledge of ARG fates during food scrap animal feeding and composting and provides a methodology for reproducible analysis.

2.2. Importance

Diversion of food scraps to agriculture is not only a sustainable practice, but in states such as Vermont it is being promoted as an alternative to meet current regulations implementing bans on food waste in landfills. In the wake of Vermont's Universal Recycling Law (Act 148) (State of Vermont 2012) and similar legislation in other states or municipalities, the fate of microbial species in food waste and residuals is under scrutiny; agricultural composts and soils represent a major contact point between the environment, animals, and humans, yet the extent of novel bacteria and associated antimicrobial resistance genes (ARGs) in co-mingled food residuals is unknown. Poultry farms may represent an increased risk, as raw food scraps can be used as feed without further processing (*e.g.*, pasteurization). Shotgun metagenomics is an alternative methodology that is not limited by culture or primer biases. Consequently, the goal of this work was to use shotgun metagenomics to assess the presence and fate of ARGs, virulence factors, and bacteria on an integrated poultry farm.

2.3. Introduction

The global crisis of antimicrobial resistance (AMR) has been attributed to the overuse and improper prescribing of antimicrobials, as well as the extensive use as growth promoters in agriculture and the slowing development of new therapeutics (Ventola 2015).

As we continue in the "post-antibiotic era", increasing pressure is placed on proper stewardship and surveillance efforts. In particular, environmental and agricultural reservoirs of resistance have been identified as key points of intervention. However, this work has focused primarily on soils, wastewater, and manures. Food wastes and residuals may be an additional important source of antimicrobial resistance genes (ARGs), yet investigation of this source is lacking.

As legislation implementing food waste composting and diversion becomes more popular, risk assessment of food wastes and residuals must be performed. Mandates such as Vermont's Universal Recycling Law (Act 148) suggests these materials might be used for agricultural feed and composting, particularly within the poultry production chain, but also for energy production on farms that utilize anaerobic digesters. Previous work has shown that both AMR microorganisms and ARGs exist in food products (Bezanson et al. 2008; Silveira-Filho et al. 2014; Sultana et al. 2014; Kevenk and Gulel 2016) at the point of consumer purchase or within households, which are also the largest producers of food wastes (EPA 2014). These co-mingled food residuals are likely to carry antimicrobial resistant bacteria and genes from multiple sources, yet their fate once they are incorporated into the farm setting is unknown.

Assessment of ARB and ARGs has been performed in similar materials, such as swine or dairy cattle manures (Zhu et al. 2013; Udikovic-Kolic et al. 2014; Wichmann et al. 2014; Ross and Topp 2015; Noyes et al. 2016; Qian et al. 2016), yet, the extent and relative importance of food scraps as a source of resistance is largely unknown. The purpose of this pilot project is to identify the range and magnitude of ARGs in food scraps received by an integrated poultry farm and composting operation. Samples of post-

consumer food wastes and residuals were collected at the source and across the farm system, from importation, to poultry feeding, to the finished composts and egg products.

Current guidelines for feeding food wastes to commercial poultry operations recognize the risk of pathogen introduction, but this is not the only avenue of resistance integration. Free-floating ARGs can be integrated into the existing microbiome of the soil, the animal, the housing environment, and/or more. This increases the prevalence of these genes in the farm environment through horizontal transfer to both closely related and divergent species of bacteria, even if the original source pathogens are eliminated. Additionally, few restrictions exist for feeding food waste to chickens, and to our knowledge, none address the potential transmission of ARGs from food waste to livestock. As there is direct contact between the "vehicle" (food waste) and the animal, a potential new source of antimicrobial resistance in the food cycle is born from implementing these practices on commercial poultry farms.

Traditional approaches to resistance monitoring or risk assessment have utilized culture-based techniques or lower-throughput culture-independent strategies such as qPCR. In this study, we utilized shotgun metagenomic sequencing to assess both the bacterial and resistance gene diversity throughout the food-scrap composting process. This technique has previously been used to investigate the resistome of sources such as manures, agricultural soils, lakes, and hospital effluents (Durso et al. 2012; Bengtsson-Palme et al. 2014; Wichmann et al. 2014; Rowe et al. 2015). Additionally, the use of cloud-based bioinformatics resources showcases the accessibility of these tools for ARG surveillance for projects of any scale.

The focus was placed on the potential impacts of human food waste composting on the poultry farm resistome, as well as the products leaving the farm for further human use as both food and material goods. The primary aim of this work was the identification and characterization of ARGs in food wastes, composts, and farm products. Additional aims include the assessment of microbial communities and potentially pathogenic species, as well as associated virulence factors from all samples to elucidate the potential mechanisms of resistance transfer within the farm environment. Finally, the relationships between these functional genes and bacterial communities were investigated to determine potential avenues for future intervention.

2.4. Materials & Methods

2.4.1. Sample Collection

Samples were collected both on-farm and at individual food scrap collection sites at a single time point in February 2017. On-farm samples included i) raw food scraps (RFSC); ii) three stages of windrow composting piles: raw compost (RWCO), unfinished compost (UFCO), and finished compost (FICO); iii) three stages of worm casting: the initial layer of substrate (TWCA), immediately after sifting (SWCA), and the packaged commercial product (WOCA); and iv) eggs from the laying hens within the barn, including outer wash as a representative of the barn environment (EGWA) and shells to represent composition upon leaving the farm (EGSH). Off-farm samples were taken as representatives from each bin present at the site, including a regional school district kitchen (SCHO), outpatient hospital kitchen (HOSP), nursing home kitchen (NURH), and grocery store (GROC) (illustrated in Figure 2.1). Additionally, a blank sample (TRBL) was included in all analysis to capture any noise generated from environmental or reagent

contamination. For each substrate type, four sterile RNA/DNA free 50 mL conical tubes (Ambion, Thermo Fisher, Waltham, MA) were filled using grab sampling across various depths and locations of on-farm piles or across bins at collection sites. Samples were collected across piles/vessels and at various depths. However, due to the time of year, much of the substrate was frozen and this impacted the ability to sample more than a few inches into the core of outdoor samples. For eggs, three eggs were taken directly from hen houses within the barn and placed into sterile containers padded with surgical gauze. All samples were transported on ice back to the University of Vermont and stored at -80 °C until further processing and DNA extraction.

2.4.2. Pre-processing and DNA Extraction

Due to the nature of food scrap samples, efforts were put into the "pre-processing" of all samples to reduce the amount of eukaryotic DNA contamination. To accomplish this, physical agitation and vacuum filtration were performed prior to DNA extraction. Briefly, 1 g of each sample was added to 10 mL sterile UltraPure water (Thermo Fisher, Waltham, MA) in a 50 mL conical Tube (Ambion, Thermo Fisher, Waltham, MA). A total of four tubes were prepared for each sample. Sterile water was warmed to 42°C to improve bacterial disruption upon vortexing. This was performed for all samples except the egg shell and egg wash. For these samples, whole eggs were placed into individual sterile Whirl-Paks with 40 mL of sterile, warmed water and gently shaken for 2 minutes. Wash material was then placed into a sterile 50 mL conical for further processing. Once washed, eggs were cracked on the edge of a sterile beaker and all interior products were discarded. Any remaining albumin was rinsed thoroughly with additional sterile water. The shell was

then crushed with a gloved hand and inserted into a sterile 50 mL conical tube with 40 mL of warmed (42°C) sterile water and agitated/crushed for 2 minutes with a sterile glass rod adapted from a previous study (Musgrove et al. 2016).

Once prepared, all sample mixtures were transferred to a multitube vortexer and shaken for 5 minutes at 1500 rpm to disrupt bacterial adhesion to any food scraps or soil particles. All samples were then filtered through a 40 μm SteriFlip (Millipore Sigma, Darmstadt, Germany) tube using vacuum filtration and combined into a single 40 mL volume per sample type. This was then centrifuged for 15 minutes at 2,000 *g* to pellet biological material. Supernatant was discarded and pellets were resuspended in 800 μL of sterile water prior to DNA extraction. Samples were stored at -20°C if not immediately used for extraction.

DNA was extracted using the Qiagen (formerly MoBio) PowerSoil kit (Qiagen, Hilden, Germany). Manufacturer's protocol was followed with the following changes. Briefly, 400 μL of the pre-processed liquid material from each sample was added to a sterile tube containing beads rather than unprocessed soil. Total DNA was eluted and stored at -20° C until quantitation and sequencing.

The concentration of DNA in each sample was quantitated using the Qubit 2.0 dsDNA BR Assay system (Thermo Fisher, Waltham, MA). The manufacturer's protocol was followed and 1 μL of sample DNA to 199 μL of working solution was used. Concentrations ranged from <0.025 ng/μL in the trip blank to 13.5 ng/μL in the finished compost, with an average of 3.7 ng/μL in experimental samples.

2.4.3. Library Preparation & Shotgun Metagenomic Sequencing

Library preparation and sequencing was performed at the UVM Cancer Center Advanced Genomics Lab (Burlington, VT). DNA quality was assessed and fragmentation was performed using the Bioanalyzer system and Covaris, respectively. A total of 2 ng of DNA from each sample was used for library preparation using the Nextera reagent kit (Illumina Inc., USA). All libraries were checked for quality using the Bioanalyzer system prior to sequencing. All 14 samples (13 samples + 1 trip blank) were sequenced via 100 bp single end (SE) Illumina HiSeq shotgun sequencing. Two lanes in total were used, from different flow cells and on different days, as technical replicates as well as to increase the total sequencing depth.

Initial sequence analysis was performed by the UVM Bioinformatics Shared Resources (Burlington, VT). This included demultiplexing (assigning reads to their sample using the barcodes from the library preparation stage), quality checking using FastQC (Andrews 2010) and storage on a remote server (VACC). Once sequences were retrieved, quality was examined using FastQC output files. Average sequence length was 107 bp and average quality was above Q 30, indicating that both lanes had high-quality sequences.

2.4.4. Sequence Analysis

The CosmosID (CosmosID Rockville, MD) software suite was used for both identification and classification of functional genes and bacterial content in all samples. Briefly, CosmosID is a cloud-based platform that uses curated reference datasets to rapidly assign metagenomic reads to the species, sub-species, and even strain level, as well as a wide array of virulence factors, antimicrobial resistance genes, and other functional databases. This is accomplished using two main algorithms, the first of which is the ‘pre-

computation phase that constructs a whole genome phylogeny tree using sets of fixed length n-mers (referred to as biomarkers) from the curated database. Once constructed, the second 'per-sample phase' searches metagenomic reads from submitted samples against the biomarker 'fingerprints' for identification. Resulting statistics are aggregated to maintain overall precision and allow for sample composition, including relative abundance estimates, frequency of a biomarker hit, total coverage of the reference sequence (Total Match %), and total coverage of unique biomarkers (Unique Match %). For this study, frequency and total reads were used to calculate further metrics for analysis.

Results of alignment to CosmosID databases Bacteria Q3 2017, Antibiotic Resistance Q4 2016, and Virulence Factors Q4 2016 were exported in .csv format for additional analysis in R (version 3.4.3). Previous studies utilizing shotgun metagenomics have noted that reads associated with reagent contamination can occur (Salter et al. 2014; Kim et al. 2017) and contributes to potential false positives within shotgun sequencing datasets. As a result, filtering was conducted by using all results from the trip/extraction blank (TRBL). Briefly, any samples with an extract match (*i.e.*, same strain or gene) or match on the same branch (*i.e.*, matched to same node within the database) to those within either TRBL sample were removed from further analysis. This strategy was used as some results may simply be rare, and occurrence in a blank rather than a read threshold allows these rare results to be conserved. Additionally, redundant results in the form of repetitive branch hits that may result from short or erroneous reads. For example, if a sample contained both a branch result for *Staphylococcus* and a more specific result of *Staphylococcus aureus*, branch results were removed so as to not artificially inflate sample

diversity. These types of removals are responsible for the majority of filtered hits, results of which are shown in Table 2.1.

Finally, an additional parameter was calculated to aid in comparative analysis between experimental samples. As each sample contained differential proportions of reads associated with eukaryotic DNA, an abundance ratio similar to gene copy/16S rRNA copy was created. The metric allowed for a better representation of the abundance of resistance genes and virulence factors by accounting for the putative bacterial load of the sample. Abundance ratios were calculated as total bacterial hits/total reads per sample and hits/ total bacterial hits and expressed as counts/bacteria in results.

2.4.5. Statistical Analysis

Analysis on filtered results were performed using R (version 3.4.3), including total genes per sample, abundance ratios, and aggregation of results by sample. Heat maps of virulence factors and ARGs were generated using the function heatmap.2 in the gplots package (v.3.0.1, Warnes 2016) and were scaled by row to normalize results by gene across samples. Calculations of sample diversity (richness, Shannon, and Simpson) were performed using the vegan package (v.2.4-6, Oksanen, 2018). The metaMDS function using Bray-Curtis distances were used for NMDS ordination of virulence genes and bacterial communities in the vegan package.

Relationships between functional genes and bacteria were assessed by Pearson's correlation coefficient and co-inertia analysis. Correlation tests were performed using the Hmisc package (v.4.1-1, Harrell 2018). Co-inertia analysis was performed using the made4

package (Culhane, 2005). Visualizations and figures were made using ggplot2 (Wickham, 2009).

2.4.6. Assessment of Functional Resistance

In addition to metagenomic sequencing, functional resistance was also assessed in a limited capacity. Aliquots (50 µL) of the pre-processed sample material homogenates were plated to various culture media and incubated under aerobic conditions at 37°C for up to 36 hours. Culture media included Mueller-Hinton agar plates containing: 1) no antibiotic, 2) Tetracycline (10 and 20 µg/mL), 3) Gentamicin (5 µg/mL), or 4) Penicillin (2 µg/mL). These drugs were chosen because they belong the classes of drugs of the ARGs found most frequently from the metagenomic data, as well as being widely used in both clinical and agricultural settings. Preliminary experiments demonstrated fungal and mold growth that overgrew individual bacterial colonies. In order to prevent fungal growth, all agar plates contained 2.5 mg of Amphotericin (Sigma, USA).

Growth of presumptive bacterial colonies on each plate type was quantified as positive or negative, and individual colonies were selected for future analyses. Individual isolates for storage and subsequent identification were passaged to trypic soy agar (TSA) plates and incubated aerobically for 24-48 hrs at 37°C. Purity of isolates on TSA was confirmed visually by evaluating growth characteristics including colony morphology, and individual colonies were selected from the pure cultures and stored at -70° C using the Microbank cryogenic system (Pro-lab Diagnostics, Ontario, Canada).

2.5. Results & Discussion

2.5.1 Sequencing and Additional Read Filtering

Total data generated, read numbers, and results of filtering are shown in Table 1. Total reads ranged from 3,753,273 to 33,835,024 excluding blanks, with an average length of 107 bp and Phred scores above Q30. Total depth and read number did not appear to significantly impact results between samples, however, as total read number is not directly associated with bacterial reads (*e.g.*, NURH on lane 1 versus EGWA or HOSP samples, which had vastly different total reads yet similar bacterial reads). Blank samples had lower total reads and reads associated with bacteria. After filtering, an average of 54 bacterial species, 7 resistance genes, and 9 virulence factors per sample were identified after filtering.

Prior studies used ARDB and Resqu databases for ARG annotation for analysis (Bengtsson-Palme et al. 2014; Jalali et al. 2015). However, source material from these studies were lake sediment and sterile swabs of paper money, respectively, which likely contain less diverse eukaryotic DNA contamination compared to food waste and compost samples; for example, when a single eukaryotic host can be identified (*i.e.*, human) those sequences can be filtered and removed, but this is an intensive process when dealing with an unknown number of plant genomes in composted materials. In order to accurately and efficiently identify both ARGs and bacteria present, CosmosID was used instead. By utilizing an algorithm based on data mining and phylogenetic approaches, rather than sequence assembly and alignment, these results were less susceptible to errors that eukaryotic sequences may have introduced during contig or genome assembly. This approach allows for better coverage of individual genes given the relatively short

sequences generated by shotgun sequencing. Additionally, CosmosID's databases are heavily curated and updated, including over 150,000 bacterial genomes and recently ranked highest in sensitivity and accuracy when compared to other popular metagenomic analysis tools (McIntyre et al. 2017).

2.5.2 Characterization and Persistence of ARGs

A total of 50 unique ARGs were found, ranging from 0 to 21 per sample, with individual gene abundance ratios ranging from 0 to 0.102 counts/bacteria. Total abundance ratios per sample, a proxy for overall "load" of ARGs, ranged from 0 to 0.431. Genes spanning 8 drug types were found, as well as ARGs regulating resistance mechanisms (Figure 2.2). Egg wash (EGWA), egg shells (EGSH), and unfinished composts (UFCO) had the most resistance genes of the on-farm samples, while the nursing home kitchen waste carried the most resistance genes of the site samples. Samples from hospital kitchen (HOSP), sifted worm castings (SWCA), and commercial worm castings (WOCA) did not have any resistance genes identified after filtering.

Most commonly found were ARGs related to Aminoglycoside (12), Tetracycline (12), and Macrolide (9) resistance. Additionally, 10 genes related to multidrug resistance were isolated in NURH samples. Resistance genes appearing in multiple samples or of particular risk to human infection are shown in Table 2.2. Of these, streptomycin resistance gene *aph(6) Id* was present in the most samples, and has been previously found in wastewater (Ng 2017) and lakes (BP 2014). Several ARGs known to reside on plasmids and mobile genetic elements were found as well, including *tetM, tetO,* and *tetW* (Roberts 2005; Luna and Roberts 1998).

Abundance ratios of all ARGs found by sample are shown visually in Figure 2.3. In addition to variation in overall load, ARGs appear to cluster by sample similarity or stage of composting. For example, clusters are composed of samples directly related to each other, such as FICO and TWCA or RWCO, UFCO, and food scrap collection sources. This pattern is observed with the presence of specific genes themselves. Tetracycline resistance genes *tetH/L/M/O/W/X* were all present in both the raw food scraps and egg samples, while genes such as *lmrD* were only present in off-farm food waste collection sites. Macrolide resistance genes, such as *mefA*/*mel*, *msrD*, and *lmrD*, were only in egg and site samples. A similar resistome profile was detected in fecal and cecal samples from broiler chickens and may represent genes shed from the animals themselves rather than food wastes (Diarra et al. 2010).

Other genes appear to be mitigated by the composting process. Tetracycline resistance genes, some of the most widespread of ARGs identified in this study, become undetected in stages. For example, *tetH, tetW,* and *tetX* were all present at the raw compost stage, with *tetW* dropping out by the intermediate stage (UFCO), and only *tetX* was present in the finished compost (FICO) and initial worm castings (TWCA). These particular Tetracycline resistance genes have been commonly found in other compost and manure samples, including swine (Zhu et al. 2013) and cattle (Noyes et al. 2016). Only one ARG, Aminoglycoside resistance gene *aph(6)-1d* was present across all stages of composting until it is no longer detected in SWCA and WOCA samples. This gene is known to reside on plasmids and integrative elements and be capable of expression in both gram-positive and gram-negative species (Jia et al. 2017), allowing for its transfer across a variety of bacterial species and perhaps explaining its persistence throughout the composting cycle.

As such, it may make an ideal candidate for use as a marker gene of plasmid transfer in future studies.

Finally, while ARGs were present in both egg samples (EGWA and EGSH) they may present a lesser risk when compared to the initial raw food scraps. Genes in these samples are typically plasmid mediated, however chromosomally encoded genes (*aph(3')-III, mefA/mel)* were present, as well as genes rarely present in pathogenic species (*aadA24, ant(9)-Ia, lnuB)*. This abundance of plasmid or transposon mediated ARGs (*aadA1, sat4A, tetO, lnuB*) suggests that ability to undergo HGT or persist in multiple host species may be the key to ARG survival throughout food waste composting. However, none of the genes present in these samples confer MDR and many are commonly associated with the soil and agricultural environments. As such, they present limited risk to recurrent clinical infections in human hosts compared to ARGs present in raw wastes. These surviving genes transferred via mobile elements do present an area for future intervention however and may be addressed in future studies through the use of additives aimed at blocking horizontal gene transfer mechanisms, such as synthetic fatty acids (Getino et al., 2015).

2.5.3 Virulence Factors: Integrases, transposons, and enabling gene transfer

Fifty-four unique virulence factor associated genes were identified, with at least one being present in every sample type. The most frequently found were the genes *intl1, sul1,* and *tnpA*. Individual abundance ratios varied from 2.02^{-6} to 0.0402 and sample averages from 0.0002 to 0.056. While less abundant than ARGs identified, the total number of genes per sample was higher; an average of 9 virulence factor genes was found per sample compared to 7 ARGs.

Visualization of abundance ratio by heatmap displayed a more diffuse pattern of virulence gene abundance compared to ARGs (Figure 2.4). Low abundance carriage of multiple genes was common, especially among EGSH, EGWA, RFSC, UFCO, and UFCO. While present in UFCO and TWCA as well, abundance of *sul1* in WOCA and SWCA was notably higher and one of only three virulence genes present in these samples.

Additional analysis using Bray-Curtis distance and NMDS plotting showed that while there is still slight separation by sample, abundance and profile of virulence genes was quite homogenous across sample type (Figure 2.5). Slight separation of worm castings, core farm, and site samples is still apparent, but these results generally recapitulate clustering shown by Figure 2.4.

Of the virulence factors detected, several key integrases and transposon regulator were identified (Table 2.3). *Intl1, tnpA* and *sul1* are commonly associated with the transfer of antimicrobial resistance (Szekeres et al.; Bennett 2008).

2.5.4 Microbial Communities, Niches, & EKSAPE pathogens

Microbial composition to the level of species or strain was accomplished using the CosmosID platform, a significant advantage over amplicon techniques. This allowed for not only the assessment of community structures and diversity, but also tracking of specific bacterial pathogens of concern.

Microbiome composition appears to be more strictly clustered than that of virulence genes, and differed not only between farm and collection sites, but specific locations on the farm as well (Figure 2.6) Within the farm, distinct similarity can be seen between

samples near the barn or in close contact with poultry (RFSC, EGSH, EGWA) and those at various stages of composting or vermicomposting.

In addition to compositional differences shown via NMDS ordination, several phyla appear only in vermicomposting samples (TWCA, SWCA, and WOCA). These include Thaumarchaeota, Verrucomicrobia, and Gemmatimonadetes. These have been prevalent in other vermicomposting studies (Danon et al. 2008; Neher et al. 2013; Huang et al. 2017). In particular, Verrucomicrobia was found to correlate with cured composts (Danon et al. 2008) and are promoted by earthworms (Neher et al. 2013). Other vermicomposting studies have indicated that dominant phyla may act as antagonists and help reduce pathogenic species (Hénault-Ethier et al. 2015).

In addition to shifts in phyla, specific strains and species can be tracked across samples due to the use of shotgun metagenomic sequencing. In terms of clinical infection risk, many surveillance efforts track the occurrence of ESKAPE pathogens. EKSAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* species) are responsible for the majority of nosocomial infections globally and can readily acquire antimicrobial resistance (Santajit and Indrawattana 2016; Schürch and Schaik 2017). Pathogens on this list were identified in several samples in this study but did not persist or occur in any samples that would be leaving the farm or used in agricultural land application. *Klebsiella pneumoniae* and *Acinetobacter baumanii* were both isolated from the nursing home samples but were not present in any other materials. *Salmonella enterica* was also present in food wastes from the nursing home, a species commonly causing severe food borne illness. *Staphylococcus aureus* was present in all four sites and the raw and

unfinished composts. While not identified in any sites sampled at this time, *Pseudomonas aeruginosa* and members of the *Enterobacteriaceae* family were identified in the raw and unfinished composts and raw food scraps and raw composts, respectively. However, none of these appeared in the egg samples or finished compost products, indicating they are not a pressing risk to animal or environmental health. Only *S. aureus* was able to be characterized at the strain level, with strain MV8 being present in the majority of samples (sites and raw compost, excluding the unfinished compost). This strain has been identified as sequence type (ST) 8 and containing a derivative of the SCC*mec* IV element responsible for methicillin resistance (Ramaraj et al. 2014) Other isolates of this group (ST 8) have been identified globally in cases of community-acquired methicillin-resistant *S. aureus* infections (CA-MRSA), such as USA 300 throughout the U.S. and CA-MRSA/J in Japan (Iwao 2012). The disappearance or removal below detectable levels of this strain is promising evidence for the attenuation of EKSAPE pathogens by the composting process.

2.5.5 Functional Resistance Persists

In this preliminary assessment, functional resistance was shown in 11 of 13 samples tested against three antibiotics: tetracycline, penicillin, and gentamicin (Table 2.4). The majority of functional resistance testing was consistent; however, discordant results were observed in 7 samples (shaded gray in Table 2.4). Nine of 15 discordant cases displayed resistance to antibiotics where no ARGs for that drug class were detected; the remaining 6 cases were susceptible even in the presence of ARGs. Additionally, 4 of these occurred in the SCHO sample, which had 9 resistance genes detected via shotgun metagenomics, yet

no resistant isolates were identified by selective culture to all antibiotics and concentrations tested.

Of the potential false negative sequencing results, WOCA was most prominent; this sample displayed positive growth against all antibiotics tested, despite having no specific ARGs detected in either replicate. This could be the result of false negatives within the metagenomic dataset, although phenotype-genotype discrepancy like this has been seen in previous work (Davis et al. 2011). Additionally, drug concentrations could have been suboptimal, allowing for organisms to grow even without expression of antibiotic resistance. Work is underway to identify the organism that grew on the selective plates from these samples, and to confirm their antimicrobial susceptibility profiles. In the opposite situation, where resistance genes were detected, but no growth was seen, growth conditions may the culprit. Only aerobic conditions on a single type of media were used in this screening, and additional conditions should be tested before results are ruled as false-positives. Further investigation into these findings should be performed as the comparison between metagenomic and observed clinical resistance within the same dataset has been limited at this time.

2.5.6 Mechanism over Carrier

Transfer of specific genes or species was rare between collection sites and farm samples. A total of 3 ARGs, 9 virulence factors, and 18 bacterial species were found in both a site and any on-farm material, which may indicate successful mitigation by the composting process as seen in other studies (Liao et al. 2017). However, only four

collection sites were sampled, thus, additional analyses were performed to assess the relationship between bacterial composition and persistence of antibiotic resistance genes.

Prior work has demonstrated a relationship between antibiotic resistance genes and associated sample microbiome (Bengtsson-Palme et al. 2015; Luo et al. 2017). To investigate this potential relationship, Pearson correlations between richness, Shannon and Simpson diversities, and ARG counts and diversity were performed (Tables 2.5 and 2.6). None of these proved significant however, which prompted the investigation of potential interactions between resistance genes and virulence genes facilitating gene transfer events. Co-occurrence of virulence genes and antibiotic resistance has been shown in *Pseudomonas aeruginosa* (Hwang et al. 2016) and has a stronger association than antibiotic use alone in populations of *E. coli* (Rosengren et al. 2009; Zhang et al. 2015). In the current study, this relationship between antibiotic resistance genes and virulence factors produced the only statistically significant result, with Shannon diversities of these gene categories being positively correlated (RV = 0.553, $P = 0.05$).

This relationship was further explored through co-inertia analysis. Briefly, co-inertia analysis is a multivariate method that can robustly couple tables, ecological data or otherwise, given time points or samples are shared across measured variables (Dray et al. 2003). For example, this technique has been applied to soil ecology studies, assessing patterns of syntony in samples across environmental characteristics such as pH or temperature with microbial communities or species. The main benefit of co-inertia analysis over similar techniques such as redundancy analysis (RDA) or canonical correspondence analysis (CCA), is that it is not constrained by the number of variables or observations. Thus, it is capable of measuring the global co-structure between two sets of variables

regardless of if they can be measured on a gradient. In this study, it was applied to assess the similarity between patterns of microbial communities and functional genes (ARGs and virulence genes); results are expressed on a scale of 0 to 1, with 0 being unrelated and 1 being strong patterns of covariance. The results of co-inertia analyses provided further evidence of syntony between resistance and virulence genes (RV= 0.647), compared to 0.445 between that of bacterial communities and ARGs and 0.358 between bacteria and virulence genes. Similar mechanisms of regulation and induction, such as biofilm formation, communication, and HGT have been implicated in the link between resistance and virulence genes (Schroeder et al. 2017).

These results may shed light on the dynamics of ARG transfer specifically within the composting environment; large population shifts occurred during thermophilic phases, but the genes regulating gene transfer are more consistent. Notably, in samples where no ARGs were identified (WOCA, SWCA, HOSP) fewer virulence genes were present. Both SWCA and WOCA carried only *sul1, intl1*, and *orf6* and HOSP contained *intl1, orfC, tniC,* and *tnpA.* Conversely, samples with the most ARGs (EGWA, EGSH, NURH, and UFCO) contained 26, 15, 6, and 16 virulence genes, respectively.

Alternatively, differentiation between total microbial community and so-called reservoir hosts should be explored. Wang et al. (2017), investigated this relationship using both metagenomic and metatranscriptomic data in controlled setting to elucidate the effects of composting stage on resistome profile. While resistome profiles were stable in composition, they were able to identify different bacterial of these ARGs across stages as environmental conditions changed; this succession of a core group of reservoir phyla is likely happening in food waste composting as well and may be responsible for the

relationships identified in this study. Identification of these reservoir hosts should be conducted in further sampling efforts in addition to characterization of important virulence or functional genes facilitating ARG persistence.

2.6. Conclusion

The aim of this work was to identify, characterize, and provide insight into the dynamics of antibiotic resistance genes during food waste composting. Using shotgun metagenomic sequencing, we were able to accomplish this by evaluating the microbiome, resistome, and relevant functional genes of collected samples. While limited to a single farm, these results indicate that ARGs and pathogenic bacterial species are reduced in both number and abundance during the food waste composting process, recapitulating results shown in manure composting operations and expanding knowledge of this important management practice. Notably, the relationship between virulence factors and antibiotic resistance genes should be further explored and may be key in preventing additional spread of ARGs throughout the food waste composting process and at the commercial scale. Future research should focus on expanding this work to additional farming systems and compost management styles to fully assess the associated risk, and this work provides an accessible analytical framework and baseline data to do so.

2.7. Tables & Figures

Table 2.1 Raw reads, unfiltered reads, and filtered hits for each sample. Hits refer to the total number of reads associated with each category, while total columns indicate the total number of unique matches, i.e., total unique bacteria or genes.

Sample ID	Raw Reads	Reads with Bacteria Hit	Total Bacteria	Filtered Bacteria	Reads with ARG Hit	Total ARGs	Filtered ARGs	Reads with VF Hit	Total VF	Filtered VF
EGSH_1	4,848,612	555,216	85	42	888	15	11	859	21	13
EGSH_2	3,753,273	428,965	67	33	766	15	12	675	20	12
EGWA_1	13,209,748	1,395,355	138	67	2,421	26	21	3,543	47	29
EGWA_2	10,830,163	1,155,081	113	53	1,962	21	20	3,003	42	24
FICO_1	13,829,897	1,690,557	163	54	1,645	13	10	1,479	17	13
FICO_2	11,117,824	1,378,774	121	54	1,396	12	10	1,260	17	13
GROC_1	19,628,880	598,070	107	51	463	4	3	1,405	11	7
GROC_2	14,748,726	465,153	73	33	362	3	3	1,056	9	5
HOSP_1	10,568,228	3,747,477	119	72	1,524	0	0	4,944	11	6
HOSP_2	8,466,316	3,071,119	75	37	1,331	0	0	4,057	10	6
NURH_1	33,835,024	654,859	58	22	1,196	15	15	1,425	8	5
NURH_2	25,097,582	487,950	48	17	957	14	14	1,017	7	5
RFSC_1	9,520,651	692,419	152	66	890	12	9	795	12	6
RFSC_2	7,839,471	575,086	121	52	749	11	9	614	13	7
RWCO_1	6,536,095	422,996	184	69	661	8	4	1,231	12	10
RWCO_2	5,918,271	379,633	163	62	606	7	4	1,211	8	6
SCHO_1	5,761,907	666,454	158	96	1,055	9	7	3,246	9	3
SCHO_2	6,049,062	764,125	139	74	1,192	8	8	3,388	11	3
SWCA_1	7,582,754	157,170	94	19	191	0	0	348	4	3
SWCA_2	6,475,126	132,104	67	16	161	0	0	326	3	2
TWCA_1	7,682,146	312,351	121	43	798	8	6	789	12	9
TWCA_2	6,981,512	283,197	136	43	736	10	6	709	16	9
UFCO_1	13,092,602	1,621,983	318	151	1,657	16	13	1,515	23	18
UFCO_2	10,375,658	1,300,381	229	108	1,283	12	9	1,145	19	15
WOCA_1	9,748,285	297,759	137	40	336	1	0	538	3	2
WOCA_2	7,951,244	243,048	111	33	254	0	0	433	3	2
TRBL_1	1,071,666	209,073	31	-	10,726	61	-	1,901	4	-
TRBL_2	838,122	156,928	24	-	8,194	58	-	1,422	4	-
Average	10,119,959	851,546	120	54	1,586	13	7	1,583	13	9

Table 2.2. Selected ARGs, associated samples, and known functions. Genes selected were present in multiple samples or conferred multidrug resistance (MDR).

Sample ID	Drug Class	Resistance Gene	Function
EGSH	Aminoglycoside	*aph(6) 1d*	Encodes streptomycin resistance via phosphotransferase enzyme
EGWA			Carried by plasmids, integrative conjugative elements, and chromosomal genomic islands in a variety of bacterial species (CARD)
FICO			Previously found in wastewater (Ng, 2017),
GROC			Present in both gram-positive and gram-negative species (Ramirez, 2011)
RFSC			
RWCO			
SCHO			
TWCA			
UFCO			
GROC	Macrolide	*lmrD*	Efflux pump utilizing ABC transporter (CARD; Florez, 2006)
NURH			Chromosomally-encoded efflux pump; confers resistance to lincosamides
SCHO			Found primarily in *L. lactis* and *S. linconensis*
EGSH	Macrolide	*mefA*	Motive efflux pump conferring macrolide resistance (CARD)
EGWA			Found on an operon with *mefE* and *mel*
SCHO			Found in *S. pneumoniae*
EGSH	Macrolide	*mel*	A homolog of *msrA,* acts as an ABC transporter with macrolide resistance
EGWA			Expressed as an operon with *mefA* and *mefE*
SCHO			Found in *S. pneumoniae*
NURH	MDR Efflux pump	*abeM*	MATE pump family, extrudes aminoglycosides, fluoroquinolones, chloramphenicol, and more (CARD; Su, 2005)
			Found mainly in *A. baumannii*
NURH	MDR Efflux pump	*abeS*	Chromosomally-encoded efflux pump of SMR family, confers low-level resistance to multiple drugs & dyes (CARD; Srinivasan, 2009)
			Found mainly in *A. baumannii* , but present in *K. pneumoniae*
NURH	MDR Efflux pump	*adeF*	Complex of adeFGH operon; acts as RND efflux pump (CARD; Coyne, 2010)
		adeG	Confers resistance to fluoroquinolone, tetracyline, tigecycline, chloramphenicol, clindamycin, trimethoprim, and sulfamethoxazole
		adeH	Found mainly in *A. baumannii*
NURH	MDR Efflux pump	*adeI*	Complex of adeIJK operon; RND efflux pump (CARD; Damier-Piolle, 2008)
		adeJ	Resistance to beta-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolone, and more
		adeK	Found mainly in *A. baumannii*
NURH	MDR Efflux pump	*emrD*	Efflux pump transporter from the MFS;; mainly found in *E. coli* (CARD; Yin, 2006)
EGWA	Sulphonamide	*sul2*	Confers sulfonamide resistance via target replacement (CARD; Daly, 2005; Skold, 2001)
FICO			Present in wide range of gram-negative bacteria
RFSC			Notably present in *A. baumannii* , *K. pneumoniae* , and *S. enterica*
RWCO			
TWCA			
UFCO			
EGWA	Tetracycline	*tetH*	Tetracycline MFS efflux pump (CARD; Roberts, 2005)
FICO			Commonly linked to *sul2* and *strAB*
RFSC			Expressed in many gram-negative species, including *A. baumannii*
UFCO			Plasmid encoded, associated with tetR on pAST2 plasmid
EGSH	Tetracycline	*tetM*	Ribosomal protection protein conferring Tetracycline resistance; found on transposable elements (CARD; Akhtar, 2009)
EGWA		*tetO*	Found on conjugative plasmids (Luna, 1998)
RFSC			Associated with erythromycin resitance gene *ermB*
EGSH	Tetracycline	*tetW*	Ribosomal protection protein conferring Tetracycline resistance; present in both conjugative and non-conjugative elements
EGWA			Present in genera associated with the gut (Scott, 2000)
RFSC			Has been found in *C. difficile* (CARD)
UFCO			
EGWA	Tetracycline	*tetX*	Resistance to all clinically relevant tetracycline via an oxidoreductase activity that inactivates the drug (CARD; Volkers, 2011; Yang, 2004)
RFSC			Found in anaerobic bacteria, particularly members of the genus *Bacteroides*
TWCA			
UFCO			

Table 2.3. Selected virulence genes, associated samples and organisms, and known functions. Genes selected were present in multiple samples.

Sample ID	Associated Organism	Virulence Gene	Function
EGSH EGWA FICO HOSP RFSC RWCO TWCA UFCO	*P. aeruginosa*	*intI1*	Integrase & resistance gene marker (Murshed, 2010 Widely implicated in the spread of AMR Has been detected in environmental phages, including soils and farms (Anand, 2017)
EGSH EGWA FICO HOSP RFSC RWCO SWCA TWCA UFCO WOCA	*V. cholerae*	*intI1*	
EGWA FICO RWCO SWCA TWCA UFCO WOCA	*K. pneumoniae*	*orf6*	Contains domains related to cellular activities, such as membrane fusion, proteolysis, and DNA replication (Yu, 2005)
EGSH EGWA FICO RFSC RWCO SWCA TWCA UFCO WOCA	*P. mirabilis*	*sul1*	Linked to other genes carried on the same integron (Antunes, 2005) Associated with presence of *aadA1* (Rosengren, 2009) Associated with resistance genes in poultry meats (Ahmed, 2016)
EGWA FICO RFSC TWCA UFCO	*P. multocida*	*tetR*	Transcriptional regulator of AMR (Ramos, 2005)
EGSH EGWA FICO RFSC UFCO	*P. multocida*	*tnp*	Encodes transposase activity
EGSH EGWA FICO HOSP RFSC RWCO TWCA UFCO	*K. pneumoniae*	*tnpA*	Transposase gene Linked to strains carrying multiple ARGs (Carnelli, 2017)
EGWA FICO GROC HOSP NURH RWCO SCHO	*P. mirabilis*	*tnpA*	

Table 2.4. Summary of the results of functional resistance assays. Grey boxes indicate results discordant with shotgun metagenomic data. N/A means the sample wasn't tested against that antibiotic.

Sample	Tetracycline 10 µg/mL	Tetracycline 20 µg/mL	Penicillin 2 µg/mL	Gentamicin 5 µg/mL
RFSC	+	+	+	+
RWCO	+	+	+	-
UFCO	+	+	N/A	N/A
FICO	+	+	+	N/A
TWCA	+	+	+	N/A
SWCA	N/A	N/A	+	-
WOCA	+	+	+	N/A
EGSH	+	-	-	N/A
EGWA	+	+	-	N/A
HOSP	-	-	-	-
GROC	N/A	N/A	+	-
NURH	+	-	+	-
SCHO	-	-	-	-

Table 2.5. Summary of diversity metrics for each sample. Measurements were taken across replicates and averaged below. Richness, Shannon, and Simpson diversity were all calculated using the vegan package in R.

	Bacteria			ARG			VF	
Sample	Richness	Shannon Diversity	Simpson Diversity	Count	Shannon Diversity	Simpson Diversity	Shannon Diversity	Simpson Diversity
EGSH	37.50	2.10	0.78	12	1.84	0.80	2.24	0.88
EGWA	60.00	2.58	0.83	21	2.42	0.89	2.66	0.91
FICO	54.00	3.34	0.95	10	0.95	0.49	1.79	0.80
RFSC	59.00	2.31	0.78	9	1.80	0.81	1.30	0.66
RWCO	65.50	3.41	0.94	4	0.77	0.39	1.07	0.54
UFCO	129.50	2.87	0.81	13	1.85	0.80	2.33	0.88
TWCA	43.00	3.42	0.96	6	1.23	0.68	1.80	0.76
SWCA	17.50	2.33	0.85	0	0.00	1.00	0.74	0.45
WOCA	36.50	2.84	0.88	0	0.00	1.00	0.61	0.42
GROC	42.00	2.44	0.81	3	0.93	0.57	1.47	0.71
HOSP	54.50	1.74	0.66	0	0.00	1.00	1.14	0.56
NURH	19.50	1.37	0.50	15	2.39	0.89	0.93	0.46
SCHO	85.00	2.48	0.81	8	1.24	0.58	0.23	0.11

Table 2.6. Results of Pearson correlation testing. All tests were conducted using the Hmisc package in R; ** denotes statistical significance.

Relationship	Correlation Coefficient	p-value
Bacteria Richness * ARG Count	0.273	0.37
Bacteria Shannon Diversity * ARG Count	-0.112	0.72
Bacteria Simpson Diversity *ARG Count	-0.235	0.39
Bacteria Shannon Diversity * ARG Shannon Diversity	-0.19	0.53
Bacteria Simpson Diversity * ARG Simpson Diversity	-0.486	0.09
Bacteria Shannon Diversity * VF Shannon Diversity	0.204	0.50
Bacteria Simpson Diversity * VF Simpson Diversity	0.203	0.51
ARG Shannon Diversity * VF Shannon Diversity	0.553	0.05**
ARG Simpson Diversity * VF Simpson Diversity	-0.038	0.91

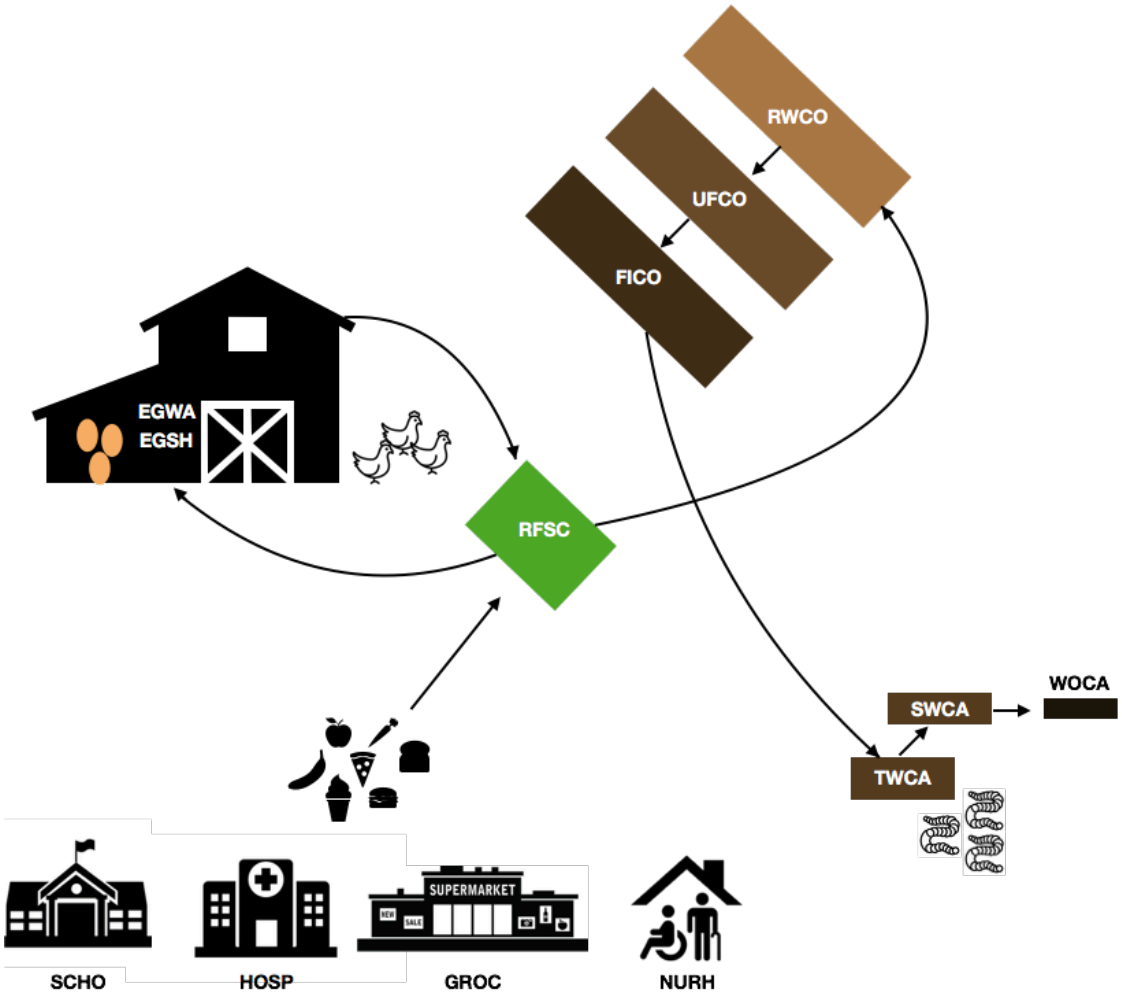


Figure 2.1. Map of sampling scheme and directionality of food scrap movement throughout the farm.

Figure 2.2 Bar chart of the total number of antibiotic resistance genes (ARGs) found by drug type and sample. In this instance, results for each duplicate were combined into a single bar shown above.

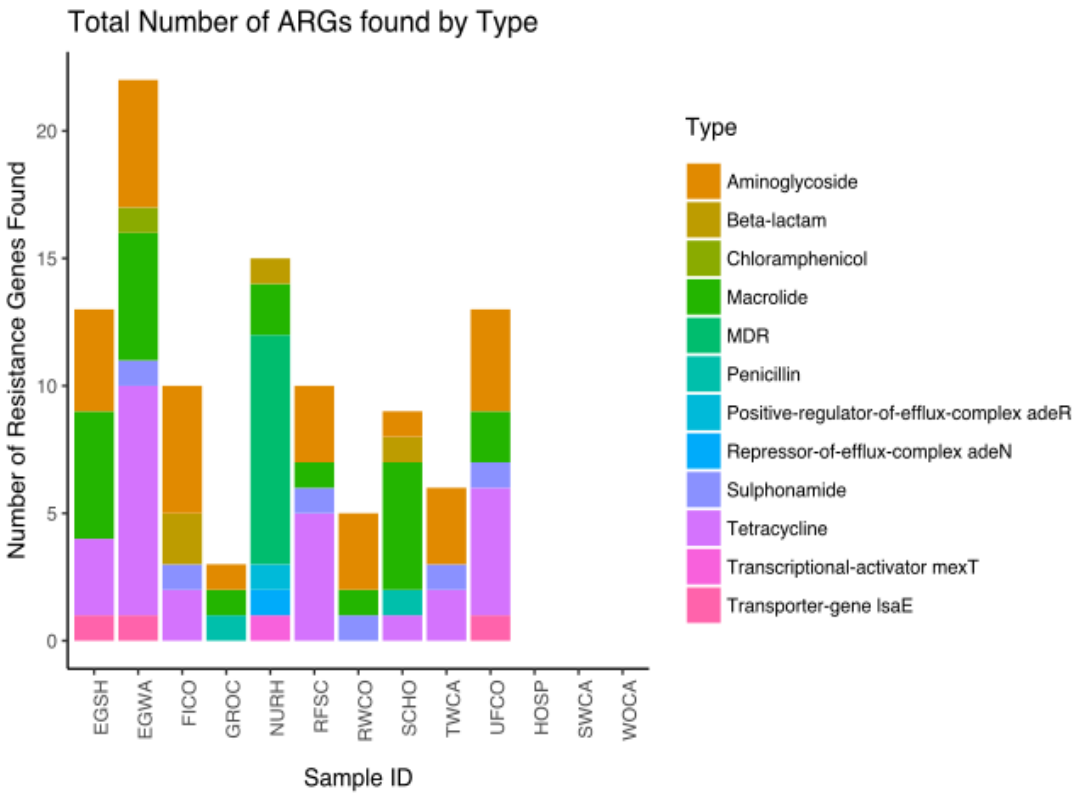


Figure 2.3. Heatmap displaying the differences in abundance ratio of ARGs between samples. Heatmap was scaled by row (individual ARGs) and created using the gplots package in R.

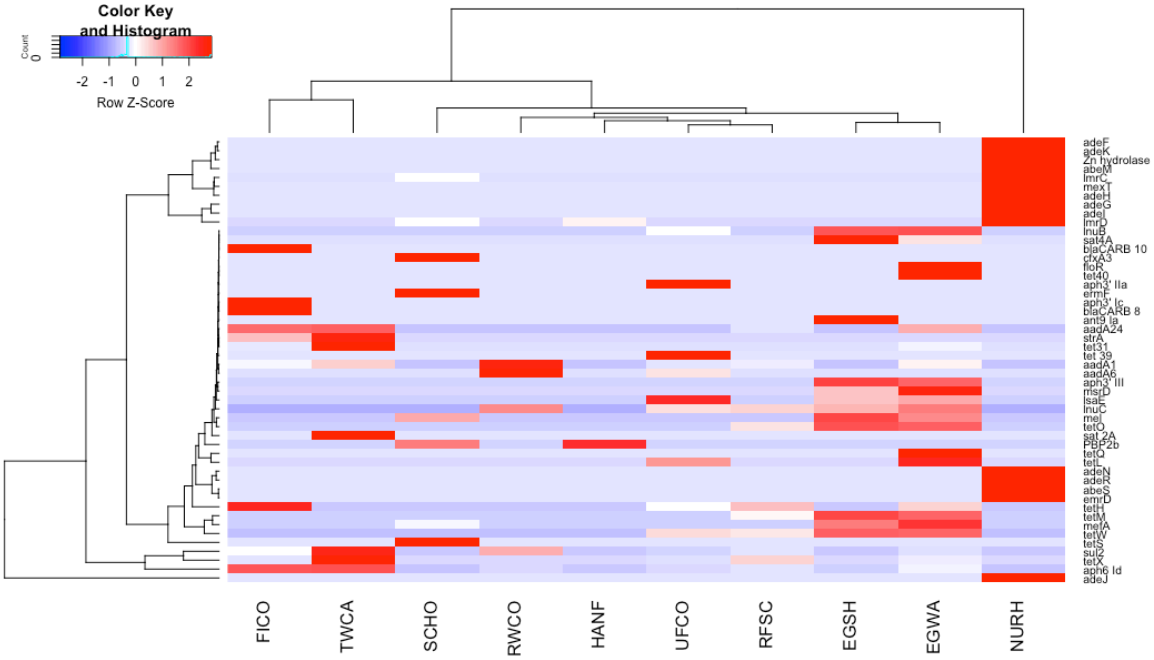


Figure 2.4. Heatmap displaying the differences in abundance ratio of ARGs between samples. Heatmap was scaled by row (individual virulence genes) and created using the gplots package in R.

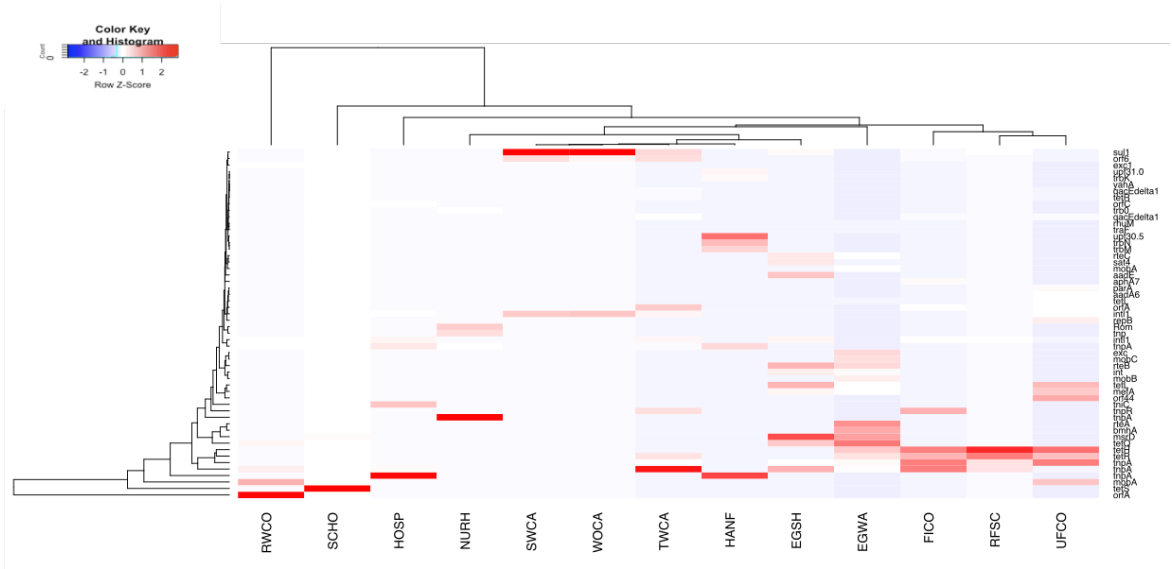


Figure 2.5. Nonmetric Multidimensional Scaling (NMDS) plot using Bray-Curtis distance of the virulence genes of each sample. Colors represent individual sample types, while shapes indicate where the sample originated (farm or off-site food scrap producer).

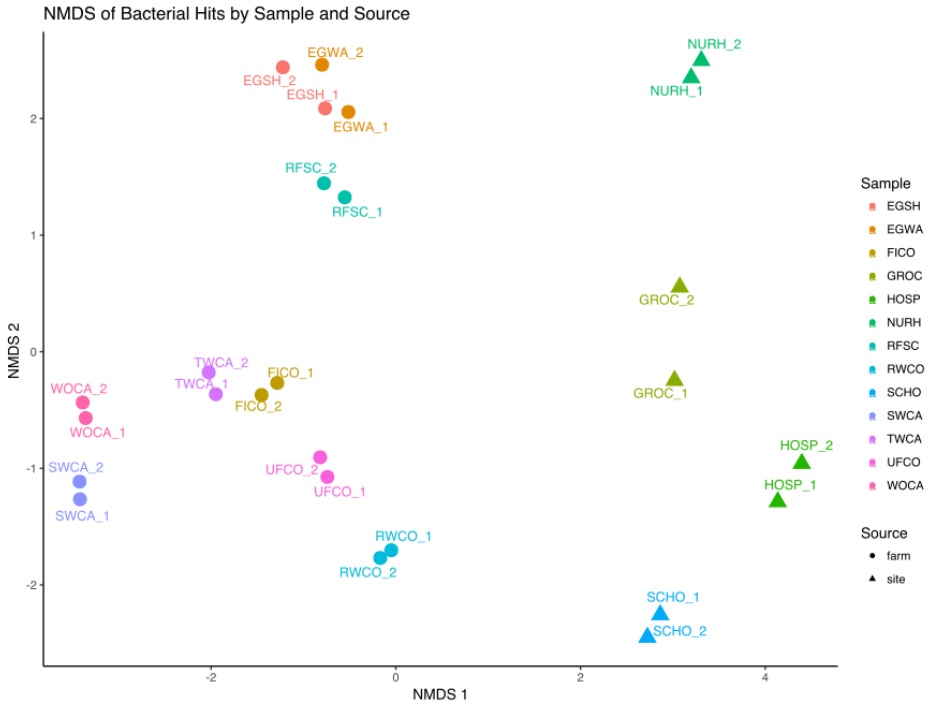
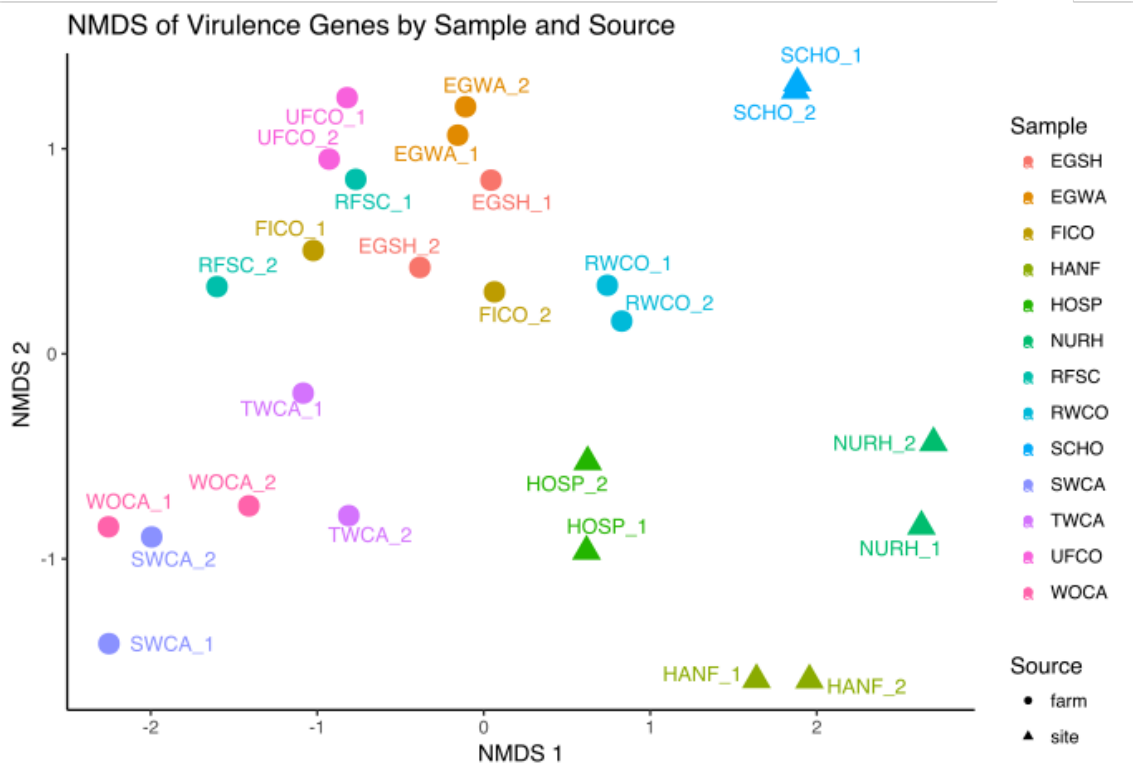


Figure 2.6. Nonmetric Multidimensional Scaling (NMDS) plot using Bray-Curtis distance of the microbiome of each sample. Colors represent individual sample types, while shapes indicate where the sample originated (farm or off-site food scrap producer).



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CHAPTER 3: GENERAL DISCUSSION

Food scrap composting is an effective solution to recapture nutrients and energy otherwise destined for landfills, mitigating the rising rate of food waste and methane emissions. However, until now the potential of these materials to act as sources or reservoirs ARGs and pathogenic species has been poorly described. This work sought to identify and characterize ARGs, virulence factors, and the microbiome of samples throughout the food scrap collection and composting process, as well as in several consumer products, in an effort to establish a baseline risk assessment for future research. There are several aspects of this work that could be adapted or improved which will be discussed in the following sections.

3.1. Effect of Sampling Strategy and Composting Method

The main limitation with the current study was the narrow sampling scheme; by enrolling a single farm the ability to make inferences to food waste composting at large is reduced. Farm-to-farm variations in soil composition, management, collection sites, and other environmental factors could all impact results and should be assessed in a more comprehensive surveillance effort.

Expansion to additional farms practicing different scales or management practices will allow for more informative guidelines for future legislation and guidelines on food waste composting.

In addition to expanding surveillance to additional farms, longitudinal analysis should be performed to better quantify the effects of season and maturity on ARG or pathogen removal. Seasonal variation of macrolide resistance genes *ermB* and *ermF* has

been observed in the South Fork Iowa River and may be the result of manure application timing or other external factors (Luby et al. 2016; Rieke et al. 2018). Seasonal variation in fungal and bacterial communities in soils (Koranda et al. 2013; Voříšková et al. 2014; Knapp et al. 2018) and ARG transport in rivers (Knapp et al. 2012) has also been observed. Sampling for the present study was conducted in February during a period of intense cold, which may have significantly impacted results.

Aging and curing of individual batches of compost may also be a critical factor. In a typical windrow operation, it takes 8-9 months from start to finish to generate a mature product in Vermont (Vermont Agency of Natural Resources 2015). While serial sampling of a single batch of food scraps at each step in the process would be ideal, controlling external factors on the farm throughout this length of time would be difficult. Alternatively, *in vitro* composting bins could be established to test single batches in a more controlled setting. A similar methodology was successfully used to test small-scale composting and anaerobic digestion of cattle manures (Williams 2016) and could be expanded upon to include spiking these materials with pathogens, plasmids containing resistance genes, or other markers to more accurately assess their dynamics over time. Additionally, the implementation of a qPCR approach for these markers would also enable an accurate assessment of gene copy number that is difficult, if not impossible, by shotgun metagenomic sequencing alone.

In addition to the effects of time and compost maturity, compost management techniques should also be tested. Past research has shown that bacterial community composition can vary greatly due to compost recipe, method, temperature, and time (Neher et al. 2013; Pruden et al. 2013; Liao et al. 2017). Further research should be conducted to

see if the same holds true for ARG composition and abundance, with a specific focus on how compost management can be used as a tool for optimal mitigation. Liao et al. (2017) demonstrated that temperature had a significant effect on mobile genetic element and ARG abundance in sewage sludge composts, and this should be examined in food wastes as well. Other physical characteristics, such as the addition of surfactants, have shown promise as a tool for more complete removal of ARGs from manure composts (Zhang et al. 2016b), and may be a useful tool for food wastes as well.

3.2. Impacts of DNA Extraction Method

Choice of DNA extraction method can significantly impact results of bacterial sequencing studies. Differential cell lysis, reagent contamination, and total input DNA have are all factors in microbiome studies. Desneux and Pourcher (2014), demonstrated that kit alone can significantly impact bacterial composition, especially of subdominant populations in swine manure effluents. Commercial extraction kits have also been shown to carry contaminating bacteria, deemed the "kitome" that may be passed on in shotgun metagenomic studies (Salter et al. 2014; Kim et al. 2017). Salter et al. (2017) also showed the serial dilution and PCR cycle can impact results, with contaminating reads taking over in samples originating from a low biomass. Putative "low contaminant" kits can be used, such as QiAmp UCP (Kim et al. 2017; Salter et al. 2014; Kim et al. 2017), however there is no guarantee these will be available for every substrate type. A better approach may be to include the use of blanks and stringent filtering as performed in this study. While it may have removed true positives, it likely had a greater impact on the removal of laboratory or reagent contaminating sequences.

Beyond contamination, method of bacterial cell wall lysis can also have an effect on results. Many commercial kits rely on mechanical or chemical lysis, however they rarely utilize both. Chemical and enzymatic lysis can be subject to additional microbial contamination and often requires specific preservation and storage conditions (van Tongeren et al. 2011), and as a result, mechanical disruption methods are typically utilized in microbiome studies. Even within mechanical techniques, additional steps such as host depletion or addition of Benzonase can improve results upon metagenomic sequencing (Wen et al. 2016). While three DNA extraction methods were tested prior to sequencing in this study, efficacy was assessed on concentration and rudimentary estimation of Eukaryotic DNA content and addition of Benzonase in future work could prove beneficial.

3.3. Sequencing Platform Choice

Shotgun metagenomic sequencing provides an improved methodology compared to techniques such as qPCR or microarray, but even since the start of this project improved sequencing technologies and pipelines have become available and more affordable that are capable of increasing genome coverage, individual gene resolution, or decrease sequencing costs. These tools include long-read sequencers such as the Oxford Nanopore or PacBio SMRT cell and the Illumina iSeq100 and NovaSeq systems.

Long-read sequencing has been used sparingly at this point for ARG detection due to the increased costs compared to short-read sequencing, but the technologies are becoming more accessible. Recently, a combination of functional metagenomics and Nanopore MinION sequencing was used as a rapid diagnostic workflow for fecal sample analysis (van der Helm et al. 2017). Barcode demultiplexing continued to be a barrier in

their study, but despite this, reads with a mean length of 1523 bp were generated and enabled high confidence identification of ARGs. In environmental samples, PacBio sequencing enabled assembly of novel genomes from a fresh water lake (Driscoll et al. 2017). Genomic assembly will allow for greater functional characterization and association of ARGs back to a specific genome in future studies, which will in turn allow for more accurate assessment of transfer dynamics.

Even more recently, improvements from Illumina came onto the market in February 2018. The release of new flow cells and sequencers themselves are poised to improve the overall accessibility and reduce cost of similar studies in the future. The NovaSeq S4 flow cell is capable of producing 6 TB of data in as little as two days; this equates to 48 human sized genomes or 384 exomes per run (Illumina 2018). As bacterial genomes are much smaller, the potential for metagenomic sequencing is immense with coverage up to 200-300x for genome assembly. Additional flow cells for the NovaSeq system, S1 and S2, are targeted for flexibility. These cells can be run at 2 x 50, 2 x 100, and 2 x 250 bp depending on experimental needs. In contrast, the iSeq 100 is a vast improvement for accessibility. Runs generating 1.2 GB can be completed in as little 17.5 hours, rapid sequencing of organisms that can be isolated and cultured will be more attainable than ever (Illumina 2018b). The machine itself is priced at just $19,900, making it a feasible addition to smaller labs or diagnostic centers that will decrease costs associated with sending samples to external facilities.

3.4. Analysis Tools

Choice of analytical pipeline can have additional impacts, regardless of sampling, extraction, or sequencing methodology. As of early 2017, there were over 80 tools for metagenomic analysis (McIntyre et al. 2017). Reviews of popular tools have yielded mixed results, demonstrating the variability of performance even on standardized datasets (Vázquez-Castellanos et al. 2014; Lindgreen et al. 2016; Nayfach and Pollard 2016; McIntyre et al. 2017; Quince et al. 2017; Vollmers et al. 2017). Acknowledging these biases, several groups have begun efforts to standardize metagenomic analysis and methodologies. These include the Microbiome Quality Control (MBQC), Genome Reference Consortium (GRC), International Metagenomics and Microbiome Standards Alliance (IMMSA), and Critical Assessment of Metagenomics Interpretation (CAMI). These organizations represent an important step forward for metagenomic standardization, but as a whole the field has not come to a consensus at this time.

However, choice of tool is still largely dependent on the individual question and budget of the researcher. In this study, the cloud-based tool CosmosID was used for analysis. This enabled the rapid, comprehensive, and functional analysis of food waste composting samples. In contrast to other pipelines, output is generated in formats that can easily be used as input for statistical analysis, such as NMDS or co-inertia used in this study. Additionally, results are highly reproducible and version control is strictly implemented. Open-source tools are not always consistently maintained and reference databases can often become outdated without proper funding. However, within CosmosID the user cannot change individual parameters or download sequences or contigs without

additional support from the company. This limits the ability to investigate novel variants or genome assembly, and alternative tools should be used if this is the end goal.

3.5. Conclusion and Future Directions

In light of all of these challenges, this work has generated significant preliminary data and established a benchmark for the investigation of antibiotic resistance in an integrated poultry system. As the practice of composting food scraps for agricultural feed increases both from legislative mandates and global need of sustainability, surveillance of these substrates and risk assessment is critical. This work, while limited in scope, demonstrates that the overall number of antibiotic resistance genes decreases throughout the composting cycle. Additionally, remaining ARGs are deemed "less risky" to human health than those that occur in food scraps directly from their source. Finally, a lack of ARGs found in certain samples leads to its own line of questioning; is microbial community diversity, specific genera, or other genetic factors responsible for the decline? If these transmission dynamics can be elucidated, it stands to reason that these conditions could be replicated and applied commercially to limit the spread of AMR from food scraps off the farm as well. Specifically, as food scrap composting expands from the farm level to waste management facilities, different burdens and selective pressures may be present that increase the rate of horizontal gene transfer. However, if a specific microorganism, bacteriophage, or other genetic element can be isolated it may reduce AMR spread in these settings. While this work does not directly answer these questions, it lays the groundwork for such examination. At this point, composting of food scraps appears to be of low risk

and can continue as a highly sustainable and economically beneficial practice for farmers and local stakeholders.

CHAPTER 4: COMPREHENSIVE BIBLIOGRAPHY

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